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The Editorial Board of the Biological Bulletin

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THE CULTURE OF EUDIPLODINIUM NEGLECTUM, WITH EXPERIMENTS ON THE DIGESTION OF CELLULOSE

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In the fermentation which occurs in the rumen of cattle and related forms there is a significant decomposition of cellulose (Tappeiner, 1884). Microscopic examination of the rumen contents discloses a microcosm teeming with protozoa and bacteria. Certain among these are the agents responsible for the cellulose digestion. However, identification and isolation of the cellulose-digesting organisms have proved extremely difficult.

Becker, Schulz, and Emmerson (1929) and Winogradow, Winogradowa-Fedorowa, and Wereninow (1930) defaunated ruminants and found no decrease in the amount of cellulose digested in their alimentary tracts. Margolin (1930) and Westphal (1934) succeeded in growing the rumen protozoa in mass cultures for several weeks and concluded that they did not digest cellulose. On the other hand, Schuberg (1888), Braune (1914), Schulze (1924), and Trier (1926) concluded from microscopic observations that cellulose was digested, and Weineck (1934) has described microchemical tests which indicate that sugar is present around cellulose particles in the digestive sack of certain rumen protozoa.

The present investigation was undertaken in order to develop methods for growing the rumen protozoa and to obtain further evidence on their capacity to digest cellulose. Because of the similarities between the mode of life of the termite protozoa and those in cattle it seemed desirable to test on the cattle ciliates the effect of factors known to be important in culturing the termite flagellates. These factors are: the presence of cellulose, proper concentrations of the inorganic salts in the culture medium (especially sodium chloride), anaerobic conditions, a suitable pH, and the concentration of protozoa (Trager, 1934; Hungate, 1939, 1942).

Culture Experiments

1. *The Effect of Sodium Chloride Concentration*

Powdered cellulose was obtained by treating absorbent cotton for several days with a strong solution of hydrochloric acid until the cotton broke up into small particles. The cellulose particles were filtered off, washed free of chlorides, air dried, and ground in a mortar. A solution of inorganic salts in tap water was prepared as follows: 0.1 per cent $NaHCO_3$, 0.1 per cent KH_2PO_4, 0.01 per cent anhydrous $MgSO_4$, and 0.01 per cent anhydrous $CaCl_2$. Thirty milliliters of the solution were placed in each of ten 50-milliliter Erlenmeyer flasks containing a few milligrams of the powdered cellulose. Sodium chloride was added to give a series of concentrations ranging from 0.1 to 1.0 per cent in increments of 0.1 per cent. The flasks were placed in a water bath at 38° C. and an oxygen-free gas mixture containing 95 per cent nitrogen and 5 per cent carbon dioxide was bubbled through each vessel. One-half milliliter of fresh rumen contents containing numerous protozoa of many species was added to each flask during the latter part of the bubbling. The flasks were then stoppered tightly with a rubber stopper and incubated in a water bath at 38° C. They were inspected daily for protozoa by holding them up toward a ceiling light and examining the bottom with a hand lens. After three days the protozoa were dead in all flasks except the ones with 0.5, 0.6, and 0.7 per cent sodium chloride. In these a few large protozoa could be seen actively swimming about.

Microscopic examination of a sample of the culture showed that the protozoa resembled *Diplodinium* (*Eudiplodinium*) *neglectum* of which several forms were described by Dogiel (1927). Considerable variations in the size and shape of the protozoa in the laboratory cultures have been observed and individuals similar to the forms *dilobum*, *monolobum*, and *bovis* have been noted. Kofoid and MacLennan (1932) considered these forms to be distinct species of a new genus, *Eremoplastron*. However, Poljansky and Strelkow (1934) have shown that the forms, *bovis* and *monolobum*, occur in clones obtained from a single individual of the form *bilobum*. For this reason it seems preferable to include them all in the species *neglectum*, subgenus *Eudiplodinium*, as arranged by Dogiel. The cultures used for experiments on cellulose digestion contained all three forms.

The freezing point depressions of the fluid portions of the rumen contents of two cows were determined, the values obtained being 0.54 and 0.59° C., respectively. The freezing point depression of the inorganic solution containing 0.6 per cent sodium chloride was found to be

0.47° C. The similarity in osmotic pressure between the optimum inorganic solution and the rumen contents suggests that the sodium chloride concentration is important because of its osmotic effect. In all the following experiments a concentration of 0.6 per cent sodium chloride was used.

2. *The Reaction of the Medium*

Six flasks containing the inorganic medium plus cellulose were adjusted with sodium hydroxide or hydrochloric acid to pH's of 7.6, 7.1, 6.6, 6.1, 5.6, and 5.1, respectively, using the glass electrode. The experimental gas (oxygen-free nitrogen and carbon dioxide) was bubbled through the cultures while the pH was adjusted. Each flask was brought to a temperature of 38° C. and then inoculated from a sample of rumen contents. Examinations for survival were made at frequent intervals. The protozoa in cultures with an initial pH ranging from 6.1 to 7.6 survived for about nine days. In the cultures with a pH of 5.6 and 5.1 the survival time was reduced to five days and one day, respectively. Later experiments have shown that the protozoa die in cultures having an initial pH of 8.0. It has also been observed that in old cultures the protozoa become inactive when the pH drops to 5.5.

In this experiment again the medium appeared to be selective for *E. neglectum* as this was the protozoan which survived longest.

3. *Size of the Inoculum*

The influence of the size of the inoculum on the longevity of the protozoa was investigated by adding varying amounts of rumen fluid to fixed quantities of inorganic medium plus cellulose. With an inoculum equal to one-third to one-half the volume of the inorganic medium the time of survival of the protozoa was much less than with an inoculum of one-half milliliter in 30 milliliters of medium. The effect was not due to exhaustion of the cellulose substrate which was present in excess. It may be accounted for by the accumulation of metabolic products which reach a toxic concentration sooner with a large than with a small inoculum. Toxic metabolic products are of particular importance where fermentative organisms such as the cattle protozoa are studied, for under anaerobic conditions acids such as formic, acetic, and lactic are formed in relatively large quantities. Slight concentrations of these acids are often toxic.[1]

[1] Levine and Fellers (1940) have reported upon the inhibiting effect of acetic acid on *Aspergillus niger*. The rapid death of Colpidium, noted by Hall (1941) when exposed to an acetate buffer can be explained as due to the toxicity of the acetic acid present. Elliot (1935) noted that acetic and butyric acids were toxic to the proto-

When very small inocula are used the protozoa often die within a few hours. The factors causing this have not been fully analyzed but death is probably due to toxic materials in the medium, among which oxygen may be mentioned. With larger inocula the combined protoplasts of the protozoa can absorb without permanent injury quantities of toxic materials that are fatal to one or a few organisms.

4. *Composition of the Organic Portion of the Medium*

Cellulose was used as a source of carbohydrate in the present experiments, since it was desired to study primarily any protozoa in the rumen which might be able to utilize this material. When ***Eudiplodinium neglectum*** was transferred from an initial culture of inorganic medium plus cellulose to a subculture of the same medium, no growth occurred. Evidently growth in the original culture was supported by materials in the inoculum, but in subcultures these materials were no longer present in sufficient quantity. In particular, it seemed probable that nitrogenous substances needed to be included in the medium.

Addition of 0.1 per cent ammonium sulfate did not improve conditions. Beef extract (0.003 per cent) and peptone (0.005 per cent) were added but subcultures still failed to show growth. Filtered rumen fluid was then included with the inorganic solution plus cellulose, and with this medium successful subcultures were obtained. Apparently the rumen fluid supplied requirements of the protozoa that were not met by beef extract and peptone. This is in agreement with the results of Westphal (1934) who concluded that factors present in the rumen were required for growth by the protozoa.

Since green plant parts are an almost universal component of the food of ruminants it seemed possible that the necessary requirements might be supplied by the grass or other green food consumed by these herbivores. This was tested by adding a little grass to the inorganic medium containing cellulose, beef extract, and peptone. The grass was added in the form of a dried powder prepared by drying and grinding fresh, green, winter grass (*Bromus catharticus*). Some cultures using this medium showed growth of Eudiplodinium but not all were successful and great variations in the numbers of individuals in surviving cultures were observed. It was then found that omission of the beef-extract and peptone improved the uniformity of the growth without sacrificing numbers of protozoa. Thus, a medium containing grass

zoan, Colpidium, whereas the salts of these acids exerted no influence or were slightly beneficial. Burnett (1940) observed that the pH range for growth of *Trichomonas termopsidis* is affected by citric acid. In its presence the optimum pH is shifted in an alkaline direction.

and cellulose as the only organic constituents proved entirely satisfactory.

Since grass contains some cellulose it seemed possible that grass alone might support growth of Eudiplodinium. However, omission of the cellulose resulted in death of the protozoa after a few transfers. Later it was found that grass alone would support the growth of the protozoa if they were transferred daily, but they did not become as numerous as when cellulose was added.

Experiments were performed to determine the optimum concentration of organic materials. It was found that dependable results were obtained with concentrations of 0.04 per cent grass and 0.04 per cent cellulose. Higher concentrations supported excellent growth for a few transfers, but it was difficult to maintain an optimum acidity and considerable variations in numbers resulted.

The following routine culture method was adopted. The inorganic medium was placed in a graduated cylinder in the water bath, and the oxygen-free gas mixture was bubbled through it for 15 minutes. Twenty milliliters of this solution were added to a 50-ml. Erlenmeyer flask containing 16 milligrams of cellulose and 16 milligrams of grass. Then 20 ml. of a vigorous *in vitro* culture of Eudiplodinium were added. Oxygen was displaced by bubbling the nitrogen-carbon dioxide mixture through the cultures. The flasks were then tightly stoppered and incubated at 38° C. Although the large inoculum used in these transfers proved unsatisfactory in the case of rumen contents, it was found that the large inoculum from an *in vitro* culture gave more uniform results, and it was not necessary to observe as great precautions in removing oxygen. The rumen contents contain a much greater percentage of organic material than the *in vitro* cultures and the bad effects of large inocula of the former may be ascribed to this factor.

Each culture undergoes a series of changes. The acidity increases from an initial pH of about 7.0 to one of 6.0 at the time of transfer. At first the cellulose and much of the grass rest on the bottom of the vessel. The protozoa swim actively about; many of them remain near the grass and cellulose but they also are found in the upper layers of the culture. As fermentation of the grass and cellulose progresses these materials tend to be carried up in the liquid by the bubbles of fermentation gases and in later stages of the culture are largely collected near the surface. The protozoa become less active and accumulate on the bottom of the flask. Subcultures should be prepared at this point or earlier because the protozoa begin to die after this time. In transferring the culture it was vigorously rotated to distribute the protozoa and undecomposed

substrate evenly throughout the medium. Twenty milliliters were then transferred to a fresh flask.

Using this procedure *Eudiplodinium neglectum* was cultured from March until June, 1940, when a failure in the temperature regulating apparatus caused the death of the cultures. In October, 1940, another culture was obtained from rumen contents and at the present time it has been maintained in the laboratory for 22 months.

Two species of grasses and one legume were tested to see if they were equally suitable for culturing *E. neglectum*. Italian winter rye (*Lolium italicum*) as well as *Bromus catharticus* supported excellent growth when the dried ground grass was used but bur clover (*Medicago arabica*) prepared in a similar fashion was unsatisfactory. With the latter the protozoa were dead after two transfers. An extract of the suitable grasses obtained either by boiling one minute in the medium or by soaking over-night could not replace the particles of dried grass.

Westphal (1934) reported success in maintaining suitable acidities by using urea in the cultures. Decomposition of the urea by bacteria liberated quantities of ammonia which neutralized the metabolic acids. In the present experiments a concentration of 0.1 per cent urea produced a reaction which was too alkaline. With a concentration of 0.02 per cent the protozoa survived well, but no better growth or regulation of acidity was obtained than when the urea was omitted. It is perhaps significant that in Westphal's experiments the species of Eudiplodinium did not survive throughout the culture period, only Entodinium showing marked growth.

5. *Numbers and Division Rate of Protozoa*

The protozoa in the routine cultures transferred at two-day intervals were counted just before transferring. The flask contents were thoroughly mixed and a 0.1 milliliter sample was withdrawn and examined under a dissecting microscope. The protozoa were counted as they were drawn up into a capillary mouth pipette. This gave an accurate count in the sample. The error due to sampling was of the order of ten per cent. The counts over a 14-day period are shown in the solid lines in Figure 1.

The curve shows the concentration of protozoa in each subculture and indicates which subculture was used for further transfer. The continuous line represents the continuous culture series.

The concentration of organisms at the time of transfer was maintained throughout the period in which counts were made and therefore it may be concluded that the protozoa divided on an average of once in 48 hours. The way in which the protozoa settled during the later

stages of the culture and the fact that they died if not transferred on schedule, suggested that the cultures were less favorable for growth in the later stages. Consequently it was anticipated that a more rapid division rate would be observed if the transfers were made more frequently. In order to test this, a second series of cultures with daily transfers was run in parallel to the first.

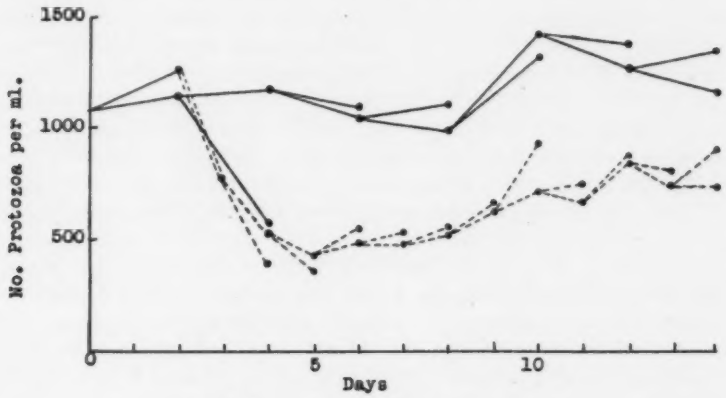

FIGURE 1. Concentration of protozoa.

The results of the second series are shown as broken lines in Figure 1. It may be seen that after an initial drop in concentration as compared with the 48-hour series the division rate increased sufficiently to maintain and even to increase slightly the concentration of protozoa at each 24-hour transfer. It follows that on the average a division occurred at least once each day.

6. *Purification of the Protozoa*

A preliminary attempt was made to grow the protozoa free of bacteria but it was unsuccessful. The wash methods which have been used to obtain pure cultures of other protozoa present special difficulties in the case of Eudiplodinium. It is necessary to maintain a suitable temperature of the wash medium and to prevent exposure to oxygen. Special anaerobic wash dishes were constructed and single protozoa were washed through as many as five transfers. They ceased moving at or before the fifth transfer and settled on the bottom. Such individuals failed to divide even though sterile grass and cellulose were present. On the other hand, in three different instances a single indi-

vidual transferred directly from a routine culture into one of the wash vessels containing sterilized cellulose and grass survived and gave rise to a thriving clone.

Physiological Characteristics

The Effect of Oxygen

The gas passed through the cultures described thus far consisted of 95 per cent nitrogen and 5 per cent carbon dioxide from which traces of oxygen were absorbed by the chromous oxygen absorbent described by Stone and Beeson (1936). The bacteria and protozoa present in the culture rapidly absorbed any traces of oxygen present initially or which might gain entrance. Thus, the protozoa grow in laboratory cultures under strictly anaerobic conditions. In their natural environment, the rumen, there is also an almost complete absence of oxygen.

Occasionally in the routine cultures the pressure in a flask due to gaseous fermentation products became so great that the stopper would be blown out, allowing free access of air to the surface of the culture. If left exposed for any length of time, the protozoa in such flasks were killed. Although presence of oxygen is the most probable cause, the death of the protozoa might also be explained as due to the growth of unfavorable bacteria, or to a change in acidity through loss of carbon dioxide.

A more convincing demonstration of the toxicity of oxygen was accomplished as follows: a gas mixture containing air and 5 per cent carbon dioxide was bubbled through samples of a thriving culture of Eudiplodinium and the effect compared with that of 95 per cent nitrogen-5 per cent carbon dioxide. Changes in acidity were the same since both gas mixtures contained 5 per cent carbon dioxide. Possible unfavorable influences exerted by aerobic bacterial growth were decreased by an experimental arrangement which permitted detection of the toxic effects of oxygen before appreciable bacterial growth could occur. The gas to be tested was brought into rapid equilibrium with the culture liquid by introducing it through a sintered glass plate of fine porosity. The plate extended across the middle of a 12-millimeter Pyrex glass tube which was immersed in the water bath to within a few centimeters of the top. The experimental gas supply was connected at the bottom of the tube, and by applying suction at the top the gas was drawn through the tube until it completely filled the part below the sintered glass. The suction was discontinued and 5 milliliters of a protozoan culture were added to the upper portion of the tube. Slight suction was then carefully applied so that a copious supply of fine bubbles of the gas passed up through the culture. At periodic intervals

the suction connection was removed and a sample of the culture withdrawn for microscopic examination.

When oxygen-free 95 per cent nitrogen-5 per cent carbon dioxide was passed through the suspension no decrease in motility of the protozoa could be detected after two hours. Using the same technique but with 95 per cent air-5 per cent carbon dioxide some of the protozoa retracted their membranelles and ceased moving after bubbling for five minutes. After ten minutes most of the protozoa were motionless and after 15 minutes no motile individuals could be observed. Similar rapid immobilization of the protozoa was obtained with alveolar gas which also contains oxygen and has a carbon dioxide content of about 5 per cent. This indicates that the protozoa are sensitive to oxygen and may be considered obligate anaerobes. They are catalase negative. When carefully washed samples of active protozoa from two cultures were treated with hydrogen peroxide no gas was evolved.

Cellulose Digestion

The fact that cellulose is a necessary ingredient of the culture medium for *E. neglectum* suggests that these protozoa use it as food. Microscopic examination shows that they ingest large quantities. However, cellulose-decomposing bacteria are present in the cultures and the beneficial effect of the cellulose as well as its ingestion might be due to its use by bacteria which in turn are fed upon by the protozoa.

Since it was not possible to grow the protozoa free of bacteria, tests for cellulose digestion by extracts of the protozoa from the crude cultures were performed. The two chief problems involved in obtaining an extract of the protozoa were (1) to raise them in sufficient numbers for extraction and (2) to free them of the cellulose-decomposing bacteria which were also present in the culture. The number of routine flask cultures of protozoa was increased to 32. Sixteen of these were used to supply protozoa for the experiments, while each of the remaining cultures was transferred to two subcultures.

Separation of the protozoa from the single bacterial cells offered little difficulty due to the large size differences. But many particles of grass and cellulose were about the same size as the protozoa and of almost the same density. Filtration and centrifugation did not accomplish a separation. After several trials the following techniques were developed and yielded protozoa almost entirely free of both large and small particles. Some of the powdered cellulose was ground with water in a pebble mill to give a very fine suspension. The upper 20 milliliters of culture medium in each of the 16 flasks used to supply protozoa was pipetted off, leaving the lower half containing the undis-

turbed protozoa. Then one-half milliliter of the fine cellulose suspension, 16 milligrams grass, and 20 milliliters of fresh inorganic medium were added, the cultures made anaerobic, and incubated. Practically all of the finely divided cellulose in these flasks was carried to the top of the medium by the fermentation gases and appeared as a cap just beneath the surface. In its rise it caught and carried upward most of the grass and debris, but the protozoa were left free and could be found swimming about near the bottom of the flask. When the debris appeared to be well separated from the protozoa, the culture fluid near the bottom of the vessel was pipetted off with as little disturbance of the surface cap as possible. It was strained through bolting silk (144 meshes to the inch) into a shallow culture dish with vertical sides. The bolting silk retained any large particles of debris but permitted the protozoa to pass through. By adding ice the filtered suspension was cooled to the point where the protozoa became immotile and settled to the bottom. The fluid above, containing bacteria and many fine particles still in suspension, was pipetted off. Fresh inorganic medium was added, the dish was agitated to wash the protozoa, and after they settled the wash solution was removed. This was repeated until the wash water showed no cloudiness and the protozoa were left relatively free of bacteria and other very small particles. The particles of debris left with the protozoa in the bottom of the dish were removed as follows. The dish was gently rotated in such a manner that the protozoa and debris were carried to the center of the dish. By further agitation of the liquid the heavier protozoa were caused to collect at the bottom of the central heap. The other material collected in clumps at the top and sides and was removed with a capillary pipette under the dissecting microscope. The remaining protozoa were almost completely free of external particles of grass and cellulose. They presented a green appearance due to the ingested grass.

Following the washing the protozoa were transferred to a centrifuge tube narrowed at the base to a small-bore tube of known diameter. They were collected in the small tube by centrifuging and their total volume was measured. Twenty to 70 microliters of washed protozoa were obtained from 640 milliliters of the original cultures.

The centrifuged protozoa were resuspended in several times their volume of inorganic medium and ground in a small mortar with a little sand. After rubbing to a fine paste additional liquid medium was added and the extract was filtered through an asbestos filter. In the first trials the final volume of the extract was about one milliliter and no evidence of cellulose digestion was observed. Karrer, Schubert, and Wehrli (1925) found that the cellulose-digesting capacity of snail

digestive juice was decreased by dilution. In later experiments the protozoan extract was kept at a volume of 0.2 ml. or less and in these tests definite evidence of cellulose digestion was obtained.

The digestion experiments were performed as follows. The fine aqueous suspension of cellulose obtained with the pebble mill was added to the enzyme extract. Small amounts of toluene were added to retard bacterial growth and the tubes were incubated at 38° C. After 24 hours the test suspensions were freed of cellulose by filtering. To the clear filtrate was added an equal volume of Benedict's solution and the resulting mixture was heated three minutes in a boiling water bath. Amounts of reducing materials were estimated by visual comparisons of the tubes. The results of a number of experiments and appropriate controls are summarized in Table II. The amount of reducing material is shown by plus signs. Several types of controls were run but due to the small volume of extract not all of these could be run at the same time.

TABLE I

Results of experiments and controls on cellulose digestion by extracts of Eudiplodinium

	Unboiled extract, cellulose	Boiled extract, cellulose	Unboiled extract, no cellulose	Boiled extract, no cellulose	Unboiled extract of debris, cellulose	Boiled extract of debris, cellulose
Expt. 1	+	−				
Expt. 2	++		±			
Expt. 3	+				−	
Expt. 4	+	−			−	−
Expt. 5	++		±	−		

Inspection of Table I shows that significant quantities of reducing materials were always formed in the tubes containing the unboiled protozoan extract plus cellulose. When no cellulose is added to the unboiled extract there is a slight reduction but it is not comparable in amount to the reduction when cellulose is present. The slight reduction in the absence of cellulose is probably due to digestion of non-reducing soluble carbohydrates in the extract. In order to determine if glycogen was present, the extract was mixed with three volumes of alcohol and the resulting precipitate was tested with iodine-potassium iodide solution. No reddish-brown color indicative of glycogen was observed whereas the precipitate did give a positive protein test with Millon's reagent. Formation of reducing materials is completely inhibited by boiling of the extract.

The debris control was performed in the same manner as the experiment except that the extract was prepared from the partially decom-

posed grass and cellulose in the cultures. This control was included because it seemed possible that the cellulose digestion observed in the experiment might be due to enzymes of bacteria ingested with the grass and cellulose. Since extracts of the debris gave no reduction it is evident that the demonstrated cellulase is not due to ingested bacteria. It may be concluded that it is elaborated by *E. neglectum*.

Some experiments were run to test the influence of pH on the activity of the cellulase. Small amounts of indicator were added directly to the extracts and the acidity adjusted to the desired point with sodium hydroxide or hydrochloric acid. A control showed that the indicator did not diminish the enzyme activity. The dilution of the extract resulting from addition of the acid or base was taken into account and proper amounts of water added to the other tubes to give the same final concentration of enzyme in the extracts at the different acidities. In these experiments the cellulase was found to be active in the pH range between 4.0 and 6.6. The greatest reduction was observed with a pH of about 5.0. Weineck (1934) noted that the endoplasm of the rumen protozoa was distinctly acid. Thus the optimum acidity for cellulose digestion by the extract is similar to the acidity at the site of cellulose digestion in the protozoa.

The nature of the reducing substances formed by the action of the Eudiplodinium enzymes on cellulose was tested as follows. An extract of the protozoa was prepared and adjusted to a pH of 5.0. Cellulose and toluene were added and the suspension was incubated 24 hours at 38.0° C. The cellulose was filtered off and to the filtrate were added 4 milligrams of anhydrous sodium acetate and 4 milligrams of phenylhydrazine hydrochloride. The tube containing the solution was stoppered and placed in the boiling water bath for 45 minutes. Yellow needle-like crystals formed while the tube was still hot and on gradual cooling of the bath these increased in number. Microscopic examination showed the rosettes, sheaves, and needles characteristic of glucosazone. They were insoluble in hot water. The crystals were transferred to a slide on which were also some crystals of pure glucosazone. The slide was heated on a hot stage on the microscope and the melting point determined. The temperature was followed by means of a thermocouple. Both groups of crystals melted at 207° C., uncorrected.

Weineck (1934) postulated that cellobiose occurred as an intermediate product in the breakdown of cellulose to glucose by the rumen protozoa. If this is true the protozoan extract should be expected to show a cellobiase action. Tests for cellobiase were run in the same manner as those for cellulase except that cellobiose was substituted for the cellulose and the products were tested with phenylhydrazine in-

stead of by reduction. The extracts were adjusted to a pH of 5.0 and the control was heated to boiling. After 24 hours of incubation sodium acetate and phenylhydrazine hydrochloride were added to experimental and control tubes and they were heated in the water bath. Copious crystals resembling glucosazone formed in the experimental tube before cooling but none were present in the control. When transferred to hot distilled water these crystals did not dissolve. Typical crystals of cellobiosazone appeared in the control tube on cooling and to a lesser extent in the experimental tube. These crystals readily dissolved when transferred to hot distilled water. These results indicate that *E. neglectum* contains a cellobiase enzyme and support the hypothesis that cellobiose is an intermediate step in the digestion of cellulose by this protozoan.

In the course of the enzyme extract experiments an interesting confirmation of the attachment of the nucleus to the ectoplasm was observed (Bretschneider, 1934). In the residue left after filtering off the protozoan extract the empty bodies of the protozoa could be found. The process of grinding had forced out all of the endoplasm, including the grass and cellulose particles, but the outer case of the animals was left intact. By staining with methyl green and acetic acid the nucleus could be seen still present in all of the ectoplasmic shells.

Discussion

The interior of the rumen is characterized by conditions which are not commonly met in nature and which seem to be necessary for the growth of the rumen protozoa. The success of *in vitro* culture methods is determined in large part by the extent to which they duplicate the natural conditions in the rumen. The present method for growing *E. neglectum* provides an environment which in many respects is similar to that of the rumen although there are certain differences.

The culture solution and the rumen contents are similar in being anaerobic, and in having the same temperature, pH, and osmotic pressure. The grass and cellulose supplied in the flasks represent the plant materials of the rumen but they are present in much smaller concentrations. Whereas the cultures furnish a total of 0.8 milligram of cellulose and grass per milliliter, the concentration of foodstuffs in the rumen is many times as great. This is one of the chief differences between the flask cultures and the natural environment of the protozoa. The abundant substrates in the rumen are actively fermented by the organisms present, both bacteria and protozoa, and large quantities of products are formed. These are absorbed or are neutralized by the alkaline saliva and thus are prevented from reaching a concentration

that would injure the protozoa. Under these conditions the number of protozoa normally reaches one million protozoa per milliliter (Ferber, 1928).

In the flask cultures, buffering by the medium and dilution through subculturing prevent toxic concentrations of products, but the capacity for accommodating the fermentation products is small in comparison with the rumen. Favorable conditions are maintained only if the amount of substrate is small. The limited substrate supports relatively few protozoa.

Other species of Diplodinium ingest plant parts in a fashion similar to *E. neglectum* and they, too, probably digest cellulose. Additional experiments with these forms will be necessary to determine with certainty their capacity for cellulose digestion but on the basis of the present results it appears probable that many of them can utilize this material.

Some of the protozoa in the rumen are passed on into the succeeding divisions of the stomach where they are digested and absorbed. Their importance in the diet of the host can be estimated from the growth rate of the protozoa in the rumen. The division rate of *E. neglectum* is much greater than the average rate of seven per cent of fissions each day assumed by Ferber and Winogradowa-Fedorowa (1929). Westphal's experiments (1934) also indicate more rapid division, namely, once every 12 hours for Entodinium. It is probable that the average division rate of the rumen protozoa is about that of *E. neglectum* since the latter is one of the medium-sized species. According to this assumption, the rumen protozoa, if none were removed, would each day double their weight. However, some of the protozoa are passed more or less continuously into the succeeding portions of the stomach and do not divide. Consequently, during one day, fewer protozoa are produced than if all were retained in the rumen.

In order to estimate the number of protozoa supplied to the ruminant let it be assumed that the excess protozoa are continuously removed from the rumen so that the number left is the same at all times. The rate of fission of these latter would determine the number of excess protozoa formed.

The formula expressing the number of organisms, N, produced in time t through fission by an initial number, N_0, with a division rate of r is

$$N = N_0 2^{rt}.$$

Differentiating, we obtain

$$\frac{dN}{dt} = Nr \log_e 2 = 0.69Nr.$$

When r equals unity, as with a division rate of once each day, the number of organisms produced in one day is $0.69N$. Thus, the protozoa in the rumen produce daily about 69 per cent of their weight of rumen protozoa. Ferber and Winogradowa-Fedorowa (1929) calculated that 2 per cent of the protein requirements of the sheep were supplied in the form of protozoa, assuming that 7 per cent of the protozoa were used each day. With the value of 69 per cent, as calculated from the division rate of *E. neglectum*, it is evident that the protein supplied to the ruminant in the form of protozoa constitutes more nearly 20 per cent of its nitrogen requirement. Schwarz and Bienert (1926) concluded from their investigations that the protozoa in the caecum of the horse supplied their host with one-fourth to one-third of its required protein. These estimates show that the protozoa compose an appreciable fraction of the nitrogenous diet.

The older work of Wilsing (1885) and Henneberg and Stohmann (1885) indicated that the products of the cellulose fermentation in the rumen are absorbed by the host. Thus, the protozoa are concerned with the carbohydrate as well as the protein nutrition of the ruminant. Whether their total role is helpful or harmful cannot be determined as yet. However, the data at hand do indicate that some of them are helpful.

The experiments of Becker, Schulz, and Emmerson (1929) and Winogradow, Winogradowa-Fedorowa, and Wereninow (1930), showing that defaunated animals digested about as much cellulose as those with protozoa, do not disprove a cellulolytic action by the protozoa. The experiments show that the protozoa are not essential for cellulose digestion, but the probable explanation is that some of the bacteria in the rumen also digest cellulose and in the absence of the protozoa they become sufficiently numerous to carry the entire burden. When the protozoa are present, some of them also participate in cellulose digestion. Since the ruminant is unable to digest cellulose it is evident that any cellulose digestion by the protozoa is helpful and in this respect the cellulose-digesting forms are definitely symbionts.

Summary

A successful method for culturing a cattle ciliate, *Eudiplodinium neglectum*, has been developed. A medium containing grass and cellulose in addition to inorganic salts has supported continuous culture for a period of 22 months. The osmotic pressure of the medium must be suitable and oxygen must be excluded. The protozoa are obligate anaerobes.

The protozoa have been grown in sufficient numbers to permit preparation of an enzyme extract which is shown to be active in cellulose digestion. The optimum acidity for action of the cellulase is about pH 5.0, a reaction similar to that in the endoplasmic sack of the protozoa. Glucose occurs as an end product of cellulose digestion and cellobiose is probably an intermediate product since cellobiase is present. Because of its cellulose-digesting capacity, *E. neglectum* is helpful to the host and the relationship between the two is one of symbiosis.

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DIGESTION OF FAT IN THE RHIZOPOD, *PELOMYXA CAROLINENSIS*[1]

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Introduction

It is well known that there is fat in the form of discrete globules in the cytoplasm of protozoa. It is, therefore, assumed that they use fat as food, but the evidence in favor of this assumption is meager. Greenwood (1886–87) maintains that digestion of fat in rhizopods is doubtful, and Nirenstein (1905) says it has not been observed in any protozoa. Dawson and Belkin (1928) injected various oils into *Amoeba dubia* and measured the diameter of the globules each day for several days. Globules of cod liver, olive, cotton seed, sperm, and peanut oil decreased in size but those of paraffin and oxfoot oil and oleic acid did not. They concluded that *Amoeba dubia* digests the former but not the latter. They (1929) repeated the experiments with *Amoeba proteus* and concluded that *Amoeba proteus* digests all the oils tested except paraffin. However, as Mast (1938) points out: "It is obvious . . . that the decrease in the size of the globules may have been due to oxidation rather than to digestion." Mast and Hahnert (1935) and Mast (1938) demonstrated, however, that fat in *Colpidium striatum* ingested by *Amoeba proteus* is broken down into fatty acid and glycerine, and that these substances pass from the food vacuoles into the cytoplasm, and there unite to form neutral fat.

The following experiments were made to ascertain whether *Pelomyxa carolinensis* digests fat in ingested organisms and fat injected into the cytoplasm.

Fat in Ingested Organisms

About one hundred pelomyxae were passed through five different 5 cc. portions of sterile culture fluid and left in 50 cc. of this fluid without food for three weeks. Then several were taken at random and tested for fat with Sudan black. No food vacuoles were found in any of them. A few had a small number of fat globules and all had

[1] The writer wishes to express his sincere thanks to Professor S. O. Mast, who suggested the problem and under whose immediate direction the work was done, for constant encouragement and invaluable aid in preparing the manuscript.

several minute granules which stained black with Sudan black and were probably fat.

The remaining pelomyxae were put into culture fluid with numerous specimens of *Colpidium striatum* and left until each pelomyxa had ingested several. The pelomyxae were then taken out, passed through five separate portions of culture fluid, and put into 50 cc. of the fluid. Some of these were immediately stained with Sudan black and Nile blue sulfate respectively,[2] and others at intervals of two hours. The results obtained follow:

Immediately after ingestion there were numerous globules in the colpidia, which stained black with Sudan black and blue with Nile blue sulfate and only a few in the cytoplasm of the pelomyxae and these stained pink with Nile blue sulfate. The former consisted, therefore, of fatty acid and the latter of neutral fat (Parat, 1927). Four hours after feeding there were still numerous globules of fatty acid in the colpidia and only a few globules of neutral fat in the pelomyxae. Six hours after feeding, the food vacuoles had divided into two or more smaller ones and all contained globules of fatty acid, but there were now numerous small globules containing a mixture of neutral fat and fatty acid in the cytoplasm of nearly all the pelomyxae. Eight hours after feeding most of the food vacuoles still contained fatty acid, but there were many small globules and several large ones containing neutral fat and fatty acid in the cytoplasm of all the pelomyxae. Ten hours after feeding only a few of the food vacuoles contained fatty acid and there were now numerous small globules and several large ones containing neutral fat and fatty acid in the cytoplasm of all the pelomyxae examined. Twenty-four hours after feeding there were no fatty acid globules in any of the vacuoles and the cytoplasm of all the pelomyxae contained a great many globules consisting of a mixture of neutral fat and fatty acid.

The results obtained indicate, therefore, that the fatty acid in the colpidia passed out of the food vacuoles into the cytoplasm of the pelomyxae and that some of it there united with glycerine to form neutral fat.

Twelve pelomyxae, taken from a thriving culture, were centrifuged[3] and cut so as to remove nearly all the globules of fatty material,

[2] Nile blue sulfate stains neutral fat pink, fatty acid blue, and a mixture of both purple (Parat, 1927).

[3] If specimens of *Pelomyxa* are suspended in gum arabic solution and centrifuged, the protoplasmic constituents stratify as follows, in order of decrease in weight: refractive bodies, vacuole refractive bodies in food vacuoles, nuclei, crystals, beta granules, hyaloplasm, contractile vacuoles, and fat. It is, therefore, easy to cut out of centrifuged specimens nearly all the fat, refractive bodies, and food vacuoles.

refractive bodies, and food vacuoles. They were then left for several hours in culture fluid to recover from the "shock" of the operation. A few were now stained with Sudan black and the rest put into culture fluid with numerous colpidia. After each pelomyxa had ingested several colpidia, they were put into sterile culture fluid and left twenty-four hours; then some were stained with Sudan black and others with Nile blue sulfate.

The results obtained with Sudan black show that before feeding there was very little fat (Figure 1*a*) in the cytoplasm of the pelomyxae but that twenty-four hours after feeding there was much (Figure 1*b*) and the results obtained with Nile blue sulfate show that the globules in the cytoplasm after feeding consisted of a mixture of neutral fat and fatty acid, for they became purple (Parat, 1927). These results, therefore, support the conclusion reached in the preceding experiment, that the fatty acid in the ingested colpidia passes out of the food vacuoles into the cytoplasm and that some of it there unites with glycerine to form neutral fat.

This conclusion is not in full accord with the results obtained by Mast (1938) in observations on *Amoeba proteus*, for he found only neutral fat in the cytoplasm. It would seem, therefore, that the metabolism of fat is not the same in *Pelomyxa* as it is in *Amoeba*. The colpidia which he used were, however, grown in a bacteria-free medium and contained only neutral fat, whereas those used in the experiments described above were not, and they contained only fatty acid. It might, therefore, be argued that the difference observed in the metabolism of fat was due to the difference in the fat used. The following experiments concern this proposition.

Injected Olive Oil

One hundred pelomyxae were starved and put into sterile culture fluid as in the preceding experiments and an olive oil droplet [4] injected into the cytoplasm of each. The pelomyxae were then separated into two equal groups, after which the diameters of the oil droplets in one group were measured daily, and specimens taken daily from the other group and stained respectively with Sudan black and Nile blue sulfate.

The results obtained in measurements of seven of the 50 pelomyxae (taken at random) are presented in Table I. This table shows that

[4] Olive oil droplets of this kind were mounted in culture fluid on a slide, covered with a cover-slip, stained with Nile blue sulfate, and examined under high magnification. They became intensely pink and, consequently, consisted largely if not entirely of neutral fat.

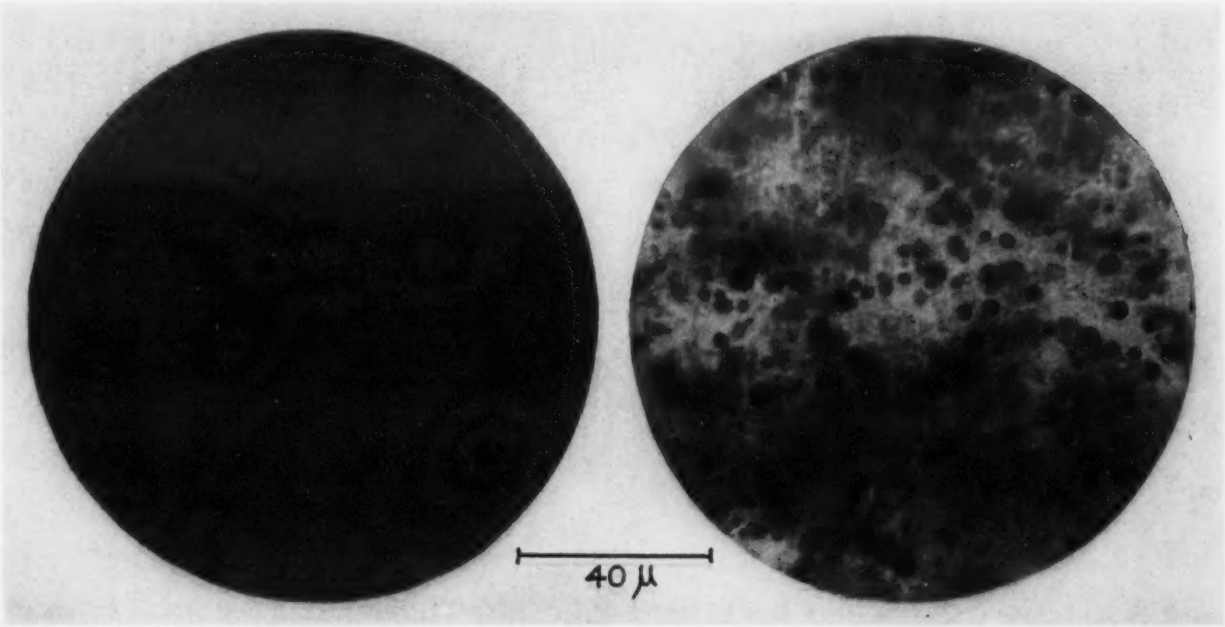

FIGURE 1. Photomicrographs of portions of specimens of *Pelomyxa carolinensis* stained with Sudan black, showing the increase in the amount of fat in the cytoplasm due to feeding on *Colpidium striatum* containing much fatty acid. A, a pelomyxa in which the fat had been removed by centrifugation; B, a centrifuged pelomyxa after having fed on *Colpidium striatum* for 24 hours; black and gray dots, fat globules. Note that there were only a few globules in the cytoplasm of A but very many in that of B.

the droplet of injected olive oil decreased in volume from day to day in all the specimens but that the rate of decrease varied with the individuals.

The observations on the stained pelomyxae show that as the injected oil droplets decreased in size, the number of fat globules in the cytoplasm increased and that these globules invariably stained purple with Nile blue sulfate, indicating that they consisted of a mixture of fatty acid and neutral fat (Parat, 1927). They show also that the injected droplets at first stained pink with Nile blue sulfate but that after they had decreased considerably in size they stained blue and that as they decreased in size they became irregular in form and a non-staining hyaline fluid formed around them.

TABLE I

Changes in the size of globules of olive oil injected into the cytoplasm of Pelomyxa carolinensis

Designation of pelomyxae	Diameters of globules in micra on successive days after injection						
	0	1st	2nd	3rd	4th	5th	6th
1	83.3	73.5	60	0			
2	34.3	19.6	14.7	0			
3	78.4	68.6	58.9	53.9	44.1	39.2	0
4	60	45	30	19	0		
5	100	40	20	0			
6	25	23	oil ejected				
7	120	60	30	0			

The fact that the droplets became irregular in form indicates that their viscosity increased greatly, and the fact that they stained blue with Nile blue sulfate indicates that they contained much fatty acid. This seems to show that at least a part of the olive oil (neutral fat) was hydrolyzed before it passed into the cytoplasm. The facts that the fat globules in the cytoplasm increased in number as the injected droplet of oil decreased in size and that these globules contained neutral fat as well as fatty acid, show that if all the injected oil was hydrolyzed a portion of it united with glycerine in the cytoplasm. They also show that the suggestion, that the decrease in the volume of injected oil observed in *Amoeba* may be due to oxidation, does not apply to *Pelomyxa*.

The color of the fat stained with Nile blue sulfate in the cytoplasm of the pelomyxae which had digested injected olive oil was repeatedly compared with that in the cytoplasm of those which had digested

fatty acid in the food vacuoles, but no difference in shade was detected. It is therefore apparent that the form in which fat is stored in the cytoplasm of *Pelomyxa*, as indicated by the staining reaction, is the same whether neutral fat or fatty acid is taken into the organism.

The results of the above two series of experiments indicate that fat metabolism in *Pelomyxa* is not the same as in *Amoeba*, for neutral fat passes from the food vacuole into the cytoplasm and is stored there in globules which in the one consist of a mixture of neutral fat and fatty acid and in the other of neutral fat. Probably all rhizopods digest fats and store them in the cytoplasm as food reserve, but the form in which they are stored is not the same.

Summary

1. If *Pelomyxa carolinensis*, which contains no fat ingests *Colpidium striatum*, containing fatty acid, the fatty acid in the food vacuoles disappears and simultaneously globules of a mixture of neutral fat and fatty acid appear in the cytoplasm of *Pelomyxa*. This indicates that fatty acid passes out of the food vacuoles into the cytoplasm and that some of it there combines with glycerine to form neutral fat.

2. If droplets of olive oil (neutral fat) are injected into the cytoplasm of pelomyxae, which contain no fat, the injected droplets gradually decrease in volume and finally disappear and numerous globules of a mixture of neutral fat and fatty acid appear in the cytoplasm. This indicates that the neutral fat in the injected droplets hydrolyzes and passes out into the cytoplasm.

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THE PERMEABILITY OF YEAST CELLS TO RADIOPHOSPHATE

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Data have been presented by Hevesy, Linderstrøm-Lang and Nielsen (1937) giving the analytical and radioactive phosphorus contents of yeast after twenty-four hours immersion in radioactive phosphorus with an external concentration of 8.7 mg. per cent phosphorus. Their data showed that inactive and active phosphorus were taken up in the same ratio as that found in the external solution, and that phosphorus uptake was negligible without sugar at 20° or with sugar at 0°. Hevesy et al. suggest either that the phosphorus content of the cell is not exchangeable, or that the cell is only permeable to phosphorus when growing. Lawrence, Erf, and Tuttle (1941) also found sugar to be essential to radioactive phosphorus uptake and demonstrated an inhibition of radioactive phosphorus uptake by fluoride ion in the presence of glucose. Further, 20 per cent of the radioactive phosphorus taken up was in nucleoprotein fractions and 80 per cent in the acid soluble fraction of yeast. They concluded that the radioactive phosphorus uptake curve of yeast could be superimposed on a curve for the metabolic rate but not for the growth curve. O'Kane and Umbreit (1942) have shown for *Streptococcus fæcalis* that when glucose is not present, organic phosphorus is lost from the cell to the medium, and that the medium suffers an increase in the concentration of both organic and inorganic phosphorus, indicating that the organic phosphorus lost from the cell is broken down extracellularly. When glucose is added, there is a marked decrease in the inorganic phosphorus both intra- and extracellularly. Later inorganic phosphorus is released. These results are in accord with theories of phosphorylating glycolysis. Ketchum (1939) using the marine diatom *Nitzschia closterium*, finds that it is possible to produce a "phosphorus debt" in this organism, when it is grown in light without phosphorus. Normally the diatom will not take up phosphorus in the dark, but when a phosphorus debt has been incurred, it can be repaid by uptake in the dark. This phosphorus uptake is independent of the external concentration of phosphorus and this suggests that the reaction is a definite protoplasmic combination with the element. This view is further substantiated by the observation that in light, phosphorus

uptake is proportional only to the number of new cells formed and not to concentration.

In order to clarify the effects of metabolic factors on phosphate transfers, the cells of *Saccharomyces cerevisiæ* were studied under conditions where the following were varied: oxygen tension, temperature, and carbohydrate supply. Measurements were then made of radioactive phosphorus (P*) and analytical phosphorus (P) taken up by the cell.

Experimental

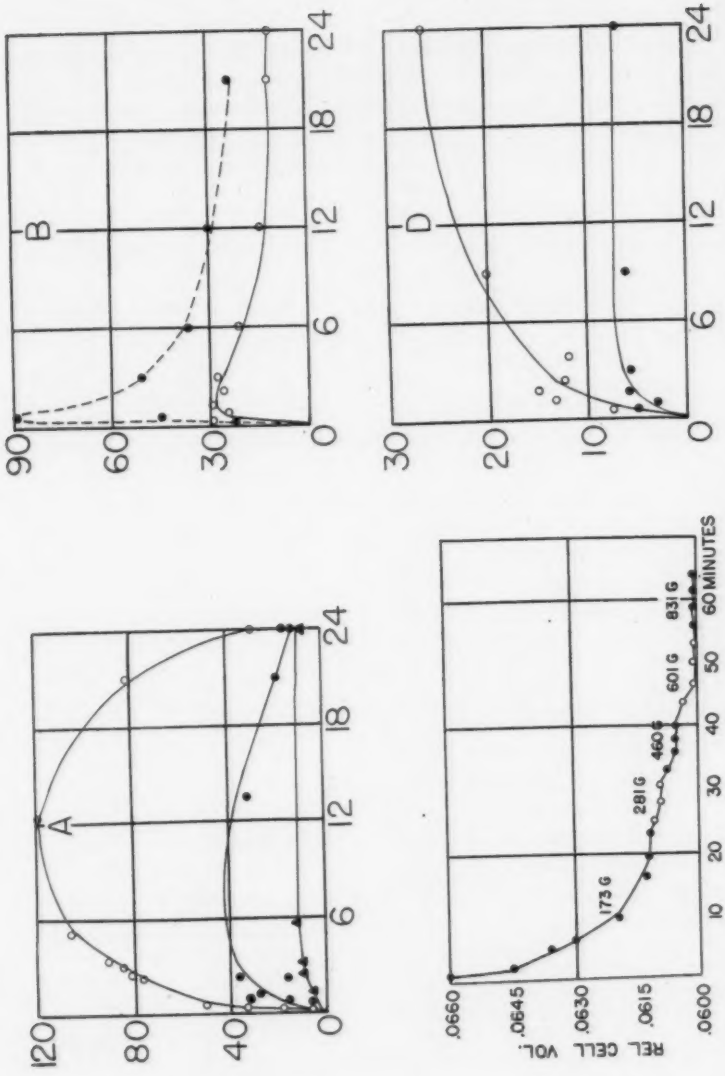
Yeast cells were obtained in cakes and freed of extraneous material by centrifugation and washing with distilled water or saline. The cells were then suspended in approximately 100 times their own volume of standard medium [1] (sugar, nitrogen, and phosphate = SNP) and the suspension was placed in a water bath at 37° C. where it was continuously aerated and stirred by a stream of bubbles of (CO–CO_2 free) O_2 gas saturated with water vapor. After 12 hours growth, the cells were again washed with fresh medium and finally suspended in the particular experimental solution desired to which had been added 5 mg. per liter $Na_2HP^*O_4$, 20 microcuries (β) per liter (μC/L).[2] In all cases pH was adjusted to 4.5 as measured by a quinhydrone electrode. This pH varied from 4.5 to 4.2 during the experiments. After the desired times of immersion in thermostats at various temperatures and at various gas tensions, a uniform sample of the suspension was obtained and centrifuged to constant volume at 830 × G, washed in either non-radioactive medium or distilled water, dried to constant weight, digested with nitric acid, evaporated to dryness, and counted under a Geiger-Müller counter.[3] Average moisture content was determined

[1] Standard Medium = 0.02M $(NH_4)_2SO_4$
(SNP) 0.03M KH_2PO_4
5.0% Brown Sugar
(NP) = 0.02M $(NH_4)_2SO_4$
0.03M KH_2PO_4
(P) = 0.02M Na_2SO_4
0.03M KH_2PO_4.

[2] The radioactive isotope P* is ${}_{15}P^{32}$; half-life, λ = 14.30 days. Na* and Br* designate the respective isotopes ${}_{11}Na^{24}$ and ${}_{35}Br^{82}$. The absolute radioactivity is given in all cases. For P*, since no γ-ray activity has been observed, the activity is compared with the β activity of Ra. For Na* and Br* their γ activity is compared with γ-radiation of Ra in equilibrium with its decay products.

[3] The various units used in radioactive work are as follows:

Term	Symbol	Quantity
Radioactivity	R	counts/min. (c/m) [As measured by a Geiger-Müller Counter]
Standard Radioactivity	R_s	c/m/gm. of solution or tissue
Specific Radioactivity	ψ'	c/m/mole of element
Relative Specific Radioactivity	ψ'_R	ψ' (tissue)/ψ' (solution bathing tissue)


A
B
D
120
80
40
0
90
60
30
0
30
20
10
0
0
6
12
18
24
.0660
.0645
.0630
.0615
.0600
REL. CELL VOL.
10
20
30
40
50
60 MINUTES
173 G
281 G
460 G
601 G
831 G

FIGURES 1a–d.

to be 86 ± 4 per cent, and no significant difference in this figure could be produced by varying the experimental conditions of growth. In experiments where hydrogen gas was used to obtain anaerobic conditions, tank hydrogen was passed through a heated combustion tube filled with copper and was distributed to the experimental solution through block tin tubing. Other connections in the system were either of glass or copper tubing. The experimental flask was so arranged that samples could be obtained without disturbing anaerobiosis.

After counting, the dried nitric acid digest was ashed in platinum with sodium carbonate, and the ash was analyzed for phosphorus by the method of Kuttner and Lichtenstein (1930). Colors were determined with a balanced cell photoelectric colorimeter and determinations in duplicate were accurate to ± 0.5 per cent.

In the use of cell volumes, as read from graduated centrifuge tubes, for the calculation of wet weight, due cognizance must be taken of the "packing fraction" or that fraction of the indicated volume of the sediment which is actually occupied by cells. In order to determine this value accurately, a 5 per cent (by volume) suspension of yeast was made up in standard medium (SNP) saturated with oxygen and held at 37° C. for one hour. Samples of this suspension were then taken (1) for measurement of cell volume by centrifugal sedimentation (mycocrit), (2) for a determination of the number of cells, using a haemocytometer chamber, and (3) for a microscopic measurement of cell dimensions. The mycocrit measurements were carried out in short graduated capillary tubes so arranged that the entire tube projected above the metal centrifuge tube holder. By the use of a stroboscope mounted above the centrifuge, the progress of the sedimentation could be followed visually.

The results of such studies are shown in Figure 1c where the mycocrit is plotted against the time of application of a given centrifugal acceleration. The curve shows that in 50 minutes the cell volume had reached a constant value equal to 0.060 of the total volume of the

FIGURE 1a. Ordinates, R_s or the standard radioactivity of yeast; abscissae, time of treatment in hours. All curves are for yeast grown in sugar, nitrogen (as NH_4+) and phosphate (SNP). Open circles, O_2 at 37° C.; solid circles, O_2 at 10° C.; solid triangles, H_2 at 10° C. All yeast suspensions were initially 5%.

FIGURE 1b. Ordinates and abscissae as above; curves are for yeast in phosphate alone, no (S) or (N). Solid circles, in O_2 at 37° C.; open circles in O_2 at 10° C.

FIGURE 1c. Ordinates, observed cell volume relative to total volume of suspension; abscissae, time of sedimentation in minutes. Numbers on the curve are the relative centrifugal acceleration.

FIGURE 1d. Ordinates, mM/L, Na^* or Br^*; abscissae, time of treatment in hours. Medium is SNP with $Na+$ concentration = 50 mM/L and Br^- = 30 mM/L, O_2 was supplied and temp. = 37° C. Open circles are Na^* and solid circles, Br^*.

suspension. The measurement of major and minor axes of 100 cells gave, as averages, the figures 6.5μ and 5.0μ respectively. Assuming a prolate spheroid, the average volume then was 88 $\pm$ 4μ^3 or 88 $\times$ 10^{-12} cm.^{3}. By actual count of the suspension, the number of cells was 6.3 $\times$ 10^8/cm.^{3}. Therefore, the actual volume occupied should be 88.0 $\times$ 10^{-12} $\times$ 6.3 $\times$ 10^8 = 0.055 cm.^{3}. Now the mycocrit gave 0.060 cm.^{3} as the volume sedimented so .055/.060 = the "packing fraction" 0.92 $\pm$ .05. This is the percentage of the volume of the mycocrit which was really cells. It is obvious that this figure is only slightly different from 1.0; however with some cells this correction can be appreciable.

Results

In Figures 1a and 1b are shown the result of some experiments in which the standard radioactivity of the yeast cells is plotted against time of immersion in the solution. The two upper curves in Figure 1a show that the uptake of radioactive phosphorus is much accelerated

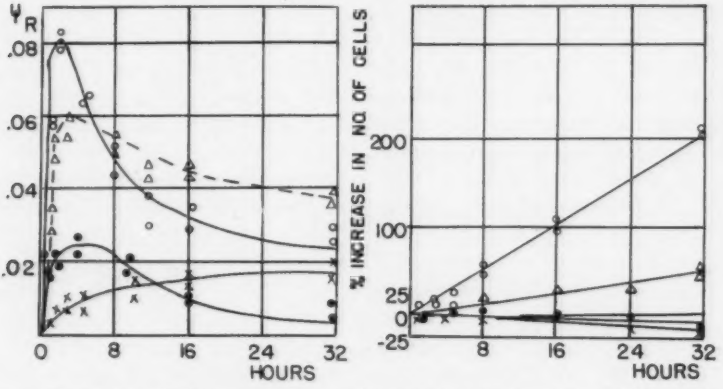


Figure 2a. Ordinates, relative specific activity; abscissae, time of immersion of cells in P* solution (SNP medium). Open circles, cells at 37° C. in O_2; open triangles, cells at 37° C. in H_2; solid circles, cells at 10° C. in O_2; crosses, cells at 10° C. in H_2.

Figure 2b. Ordinates, per cent increase in number of cells growing in SNP medium. Abscissae, time in hours. Open circles, cells growing in O_2 at 37°C. Open triangles, cells at 37° C. in H_2; solid circles, cells at 10° C. in O_2; crosses, cells at 10° C. in H_2.

by a rise of temperature from 10° C. to 37° C. while the two lower curves show that it is much inhibited by the absence of oxygen. In oxygen (upper curves) the count passes through a maximum at 6 to 12 hours and then declines. This decrease in the count indicates

perhaps a breakdown of the phosphorylated compounds due to exhaustion of the sugar in the medium.

Similar curves in the absence of sugar and nitrogen supply are shown in Figure 1b. In this case the maximum occurs sooner and disappears more quickly and must be attributed to the presence in the yeast of some residual carbohydrate capable of phosphorylation. The same marked decrease in radioactivity due to the lowered temperature is evident here and the high temperature coefficient indicates that the penetration is not due to mere physical exchange of radioactive phosphorus for phosphorus but to some chemical reaction. (Ketchum, loc. cit.)

The metabolic nature of the penetration of phosphorus is made more evident when it is compared with the penetration of radioactive sodium and bromine as illustrated in Figure 1d. In both cases the radioactivity gradually approaches a maximum value without the subsequent decline which characterizes the penetration of phosphorus.

Interpretation of Figures 1a and 1b is facilitated by Figure 2a which shows the relative specific radioactivity of the yeast phosphorus. The two curves for oxygen at 37° C. and 10° C. show a similar rapid rise to a maximum and subsequent decline. These data from Figure 2a would indicate that the decline in Figure 1a is not due to loss of both phosphorus and radioactive phosphorus but to loss of radioactive phosphorus relative to phosphorus. It could not be due to a gain in phosphorus relative to radioactive phosphorus because all the phosphorus outside the cells has a high P*/P ratio. The difference in the time relation between Figures 2a and 1a must be due to differences in the yeast used for the two experiments. A loss of radioactive phosphorus without much change in phosphorus is possible if it is the newly phosphorylated compounds which are broken down. The remainder of the cell phosphorus is evidently more permanently bound and it does not readily exchange. Figure 2b shows the relative rates of growth of yeast, as measured by cell counts, during the various experimental conditions described.

Conclusions

From the experimental evidence presented, it would seem as though the various factors responsible for the transfer of phosphorus from the external solution to the yeast cell could be enumerated as follows: a. phosphorus is used by the cell in the synthesis of relatively permanent constituents of the protoplasm; b. phosphorus is transferred in both directions across the cell membrane in connection with the phosphorylation and oxidation of carbohydrate; c. phosphorus enters the

cell by diffusion or exchange for outgoing phosphorus and is not synthesized into any organic compounds in the cell. (This is in reality the situation found in (a.) but is treated separately since the eventual fate of the phosphorus is different.) Experimentally it has been shown that radioactive phosphorus enters the cell to a relatively slight extent at 37° C. in the absence of carbohydrate. This would indicate that the amount of phosphorus required for synthesis into proteins is relatively small, since one would expect that phosphorus would be taken up for such synthetic purposes, even in the absence of sugar, if there were a need of such phosphorus. In the case of (b.) it may be stated that ample carbohydrate supply in either oxygen or hydrogen at 37° C. allows large phosphorus uptakes; the fact that this uptake is sensitive to temperature but not to oxygen would indicate that an enzymatic process of some kind was going on, and the close interdependence between phosphorus and sugar would suggest phosphorylation. As for the purely physical diffusion or exchange processes (c.), by which ions reach a concentration inside the cell which bears a direct relationship to their initial concentration outside the cell, and thus permit the computation of a "permeability constant," the data would suggest that such a permeability must at least be very low. These diffusion processes, if purely physical, should not be either temperature or oxygen sensitive to any marked degree, and hence that data for yeast in hydrogen at 10° C. should show the extent to which the phosphate ion would penetrate the cell. As can be seen from the curves, the phosphorus uptake under these conditions is very small, as compared with uptake in carbohydrate. The average phosphorus content of yeast is about 84 mM/kg., and the initial slope of the uptake curve for phosphorus in hydrogen at 10° C. is 0.25%/hr. Therefore the rate of uptake is 2.1×10^{-2} M/kg./hr.

The author wishes to acknowledge the kindness of Professor S. C. Brooks for arranging for laboratory facilities for this work. Thanks are due to Dr. Daniel Mazia for assistance with some of the experiments, and acknowledgment is also due Professor E. O. Lawrence for the supply of radioactive phosphorus, radioactive sodium, and radioactive bromine used in these experiments.

Summary

The data presented indicate that the transfer of phosphate across the yeast cell membrane is dependent almost entirely on the carbohydrate metabolism of the cell. A maximum quantity of phosphorus is transferred into the cell when high external carbohydrate concentration is maintained and when the temperature is held at 37° C. The

presence or absence of oxygen is not very important in promoting phosphorus transfer. Very little phosphorus will diffuse into yeast at 10° C. in the absence of sugar.

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THE INFLUENCE OF CERTAIN DRUGS ON THE CRUSTACEAN NERVE-MUSCLE SYSTEM

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INTRODUCTION

The physiological problems involved in the peripheral innervation of the decapod crustacea differ in many ways from those encountered in vertebrate nerve-muscle preparations. There are many structural and functional differences; the five which are most important from the standpoint of the effects of drugs will be briefly considered.

A. The number of efferent fibers innervating a crustacean muscle is very small, the largest number yet found being five, the smallest, two. In contrast to the vertebrate motor-unit concept, all of the muscle fibers in a given muscle receive a branch from each of these nerve fibers (van Harreveld, 1939b). The number of fibers innervating a particular muscle of a certain species of the decapod crustacea is constant (Wiersma, 1941a). The two muscles used in the present investigation, namely the opener (abductor of the dactylopodite) and the closer (adductor of the dactylopodite) of the cheliped of *Cambarus clarkii*, receive two and three nerve fibers respectively.

B. In contrast to the single nerve ending usually found for each muscle fiber of the vertebrates, the crustacean muscle fiber receives many branches of each of the nerve fibers innervating that muscle, and the nerve endings are well distributed over the entire length of the muscle fiber (van Harreveld, 1939a). From this and other evidence it has been concluded that the spread of excitation over the crustacean muscle fiber is due not to muscular but to nervous conduction. The contraction, then, is the sum of the many local contractions; the muscle action potential, the sum of the local action potentials set up at the individual nerve endings. (For a more complete discussion see Wiersma, 1941b.) It is doubtful, in fact, that direct muscle stimulation is possible in the crustacea to any extent greater than a local contraction at the electrodes (van Harreveld, 1939c).

C. Unlike the skeletal muscles of vertebrates, most of the leg muscles in the crustacea can contract in several ways. This was first

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observed by Richet (1879) and was subsequently confirmed by a number of authors (Lucas, 1917; ten Cate, 1927; Blaschko *et al*, 1931; Wiersma, 1933; Wiersma and van Harreveld, 1934; Knowlton, 1942). In 1936, however, van Harreveld and Wiersma showed that for the closer of the claw of the crayfish, *Cambarus clarkii*, stimulation of one (the thicker) of its two motor neurons (prepared as single fibers) invariably produces a "twitch" contraction, whereas stimulation of the other (the thinner) produces always a contraction of the "slow" type. The muscle action potentials resulting from stimulation of the prepared fibers of such a doubly motor innervated muscle are also of two distinct types. That the contractions of the two types occur, indeed, in the same muscle fiber, as would be expected from the anatomical innervation, has been established. By direct microscopic observation of small groups of muscle fibers in the closer of Cambarus, van Harreveld (1939c) found that stimulation of either of the two motor axons separately produces contraction in the same muscle fibers.

D. Whereas in vertebrate striated muscle a single nerve impulse causes a maximal twitch contraction, most crustacean muscles do not show any mechanical response unless a number of impulses reach the muscle within a limited time interval. This need for facilitation is characteristic of all crustacean slow systems, and appears, although to a lesser extent, in most fast ones (see Wiersma and van Harreveld, 1938). The fast system of the closer of Cambarus is, in this respect, an outstanding exception, since here a single nerve impulse causes both a maximal muscle action potential and a twitch contraction. Repetitive stimulation of the single motor axon to the opener produces a contraction of the slow type (van Harreveld and Wiersma, 1936).

E. The presence in the crustacea of centrifugal nerve fibers whose function is specifically inhibitory has been demonstrated by their isolation as functional single axons (van Harreveld and Wiersma, 1937). The mechanisms involved in this peripheral inhibition have been subjected to considerable investigation (Marmont and Wiersma, 1938; Wiersma and Helfer, 1941; Wiersma, 1941a, b; Wiersma and Ellis, 1942). With respect to the opener muscle of the crayfish it was found that, whereas inhibition of the contraction is usually not accompanied by depression of the muscle action potentials (simple inhibition), under certain circumstances such reduction can be observed (supplementary inhibition). It was also found that inhibition is of different efficiency[2] with regard to the contractions of different

[2] By efficiency is meant the number of excitatory impulses that can be suppressed by one inhibitory impulse. This number was found to vary from three to one-fifth in different muscles (Wiersma and Ellis, 1942).

muscles. The opener of the crayfish shows a very efficient inhibitory system, whereas the inhibitor of the closer is for practical purposes without influence on either the fast or the slow contractions.

In the present investigation the effects of drugs on the fast and slow types of contraction and on inhibition have been studied. The closer and opener systems of *Cambarus clarkii* were selected because of the great dissimilarity between the fast and the slow contractions of the closer, the negligible influence of inhibition on the closer, the ease with which the opener can be inhibited, and the presence of supplementary inhibition in the opener system.

Methods

Two types of experiments were carried out involving the use of drugs; in one group the substance was injected into the animal, in the other, the drug was applied directly to the isolated claw preparation by injecting it hypodermically through the hole obtained by removing the pollex of the propodite. In additional experiments, a limited portion of the prepared nerve was treated with a solution of the drug in a narcotic chamber.

Table I

Classification of drugs as to their generally accepted pharmacological action

Group	Sympathetic	Parasympathetic	Curare-like
Drug	Epinephrine Amphetamine Hydrastinine Yohimbine 933F	Choline Acetylcholine Mecholyl Doryl Muscarine Pilocarpine Physostigmine Atropine	Curare Trimethylammonium salts Tetramethylammonium salts Brucine Strychnine
Group	**Insecticides**	**Local anaesthetics**	**Muscle drugs**
Drug	Nicotine Pyrethrum Rotenone	Procaine Diothane Nupercaine	Caffeine Digitalin Veratrine

The drugs were selected to represent a rather wide variety of known pharmacological actions, and can be grouped as 1) sympathetic drugs, 2) parasympathetic drugs, 3) curare-like drugs, 4) insecticides, 5) local anaesthetics, and 6) muscle drugs. The ones used as representatives of these groups are shown in Table I. A wide range of dosage was used in each case. In all instances, dilution of the drug

was made in the balanced physiological solution for crayfish reported by van Harreveld (1936).

The effects of these drugs on the two types of contractions of the closer muscle necessitated isolation of the single axons by the method of van Harreveld and Wiersma (1936) to permit selective stimulation of either fiber. As the inhibitor to the opener muscle does not lie in the small nerve bundle in which the motor axon is to be found, the opener preparation did not necessitate exposing the single fibers for mechanogram studies. It was simpler just to separate the small nerve bundle from the two larger ones, and avoid interaction of the closer muscle on the opener contractions by cutting the tendon of the closer. For electrogram studies, however, the single inhibitor neuron was prepared as an isolated fiber in order to avoid the muscle action potentials of the closer.

Results

Drugs lengthening the refractory period of the nerve. Bayliss *et al* (1935), working with the nerves of Maia, and Marmont (1941) with the isolated nerve fibers of the rock-lobster, Panulirus, have reported that yohimbinized nerve fibers show a most significant lengthening of the refractory period. In Cambarus a similar result is obtained; thus after injecting a saturated solution of yohimbine (1 : 20,000 according to Bayliss *et al*) into the claw, single fast closer twitches of normal height can be obtained for an indefinite period after the injection, yet if the preparation is stimulated repeatedly with shocks following each other at intervals of about one second, a twitch can be seen to drop out occasionally (Figure 1, A). Faradic stimulation (45/sec.) causes, instead of a tetanus, a series of twitches of normal magnitude which occur at a frequency of about 1/sec. (Figure 1, A). Stimulation of the slow fiber under these conditions no longer results in a contraction, nor does stimulation of the opener. The muscle action potentials of the slow closer contraction and of the opener are correspondingly absent. The muscle action potentials of the fast twitch show little effect of the drug. During faradic stimulation a series of such action potentials is usually obtained. These effects are generally found either after injection of the drug into the claw or after treating the nerve in a narcotic chamber. Sometimes, however, when the drug is injected into the claw, faradic stimulation results in a row of action potential spikes of variable frequency and magnitude, only the larger of which are accompanied by any visible contraction (Figure 1, C). This is probably due to the unequal distribution of the drug with respect to the finer branches of the nerve fibers in the muscle; part of

the nerve fiber branches have a different refractory period than those of the rest of the arborization.

It is interesting to note that stimulation through electrodes placed on the nerve or in the muscle shows the same effect on the mechanogram. In both cases a series of twitches is obtained on faradic stimulation. Only with very strong stimulation through electrodes placed on the muscle can a continuous contraction be obtained. This indicates that unless excessively strong stimuli are used, stimulation remains indirect. The contraction obtained by strong direct stimulation is usually irreversible.

Local anaesthetics and veratrine also produce a lengthening of the refractory period of the nerve, but with veratrine this is usually more or less masked by other effects which will be described later.

Local anaesthetics. Injection of drugs of this type into the claw caused a gradual diminishing of the fast contractions, and ultimately resulted in a complete excitatory failure. When the nerve was treated in a narcotic chamber, however, the twitch was either present, full size, or was entirely absent (Figure 2). This shows that in the single fast nerve fiber the impulse is either conducted or not conducted in the region beyond the chamber. The intermediate size of the contractions on injecting the muscle can be considered to result from blocking the smaller branches of the nerve fiber at different times as introduction of the drug by injection is not instantaneous and all parts in the muscle are not acted on simultaneously by an equal concentration of the drug. At first only part of the branches are blocked, but with further spreading of the drug more and more of the nerve endings are affected, and only after some time does the entire muscle show the true drugged response.[3]

[3] Since direct stimulation of the muscle is not possible in the crustacea one cannot be sure whether the drug acts on the muscle as well as on the nerve or not. In view of the completeness of the nerve block in the chamber experiments it seems likely that the action is only on the nerve. The fact that a markedly lower concentration will produce the effects when injected into the muscle than is necessary to block the nerve is due, in part, to the more readily accessible position of the nerve fiber branches, in part, to the thinner nature of the fiber.

FIGURE 1. *Cambarus clarkii*, closer muscle. The effects of a saturated solution of yohimbine hydrochloride injected into the claw. A.—Contractions of the closer muscle in response to stimulation of the fast axon. It should be noted that the fourth, seventh, eighth, tenth, and eleventh single induction shocks were without mechanical effect. B.—Stimulation of the fast fiber with single induction shocks and the slow fiber faradically. Note the complete absence of contraction during stimulation of the slow fiber. C.—Electrogram and mechanogram during stimulation (faradic) of the fast fiber. Note the higher spikes and evidence of mechanical contraction at 2 and 3. M = mechanogram, E = electrogram, S = signal of stimulus, and T = time. In A and B time is in seconds, in C, in 0.1 second.

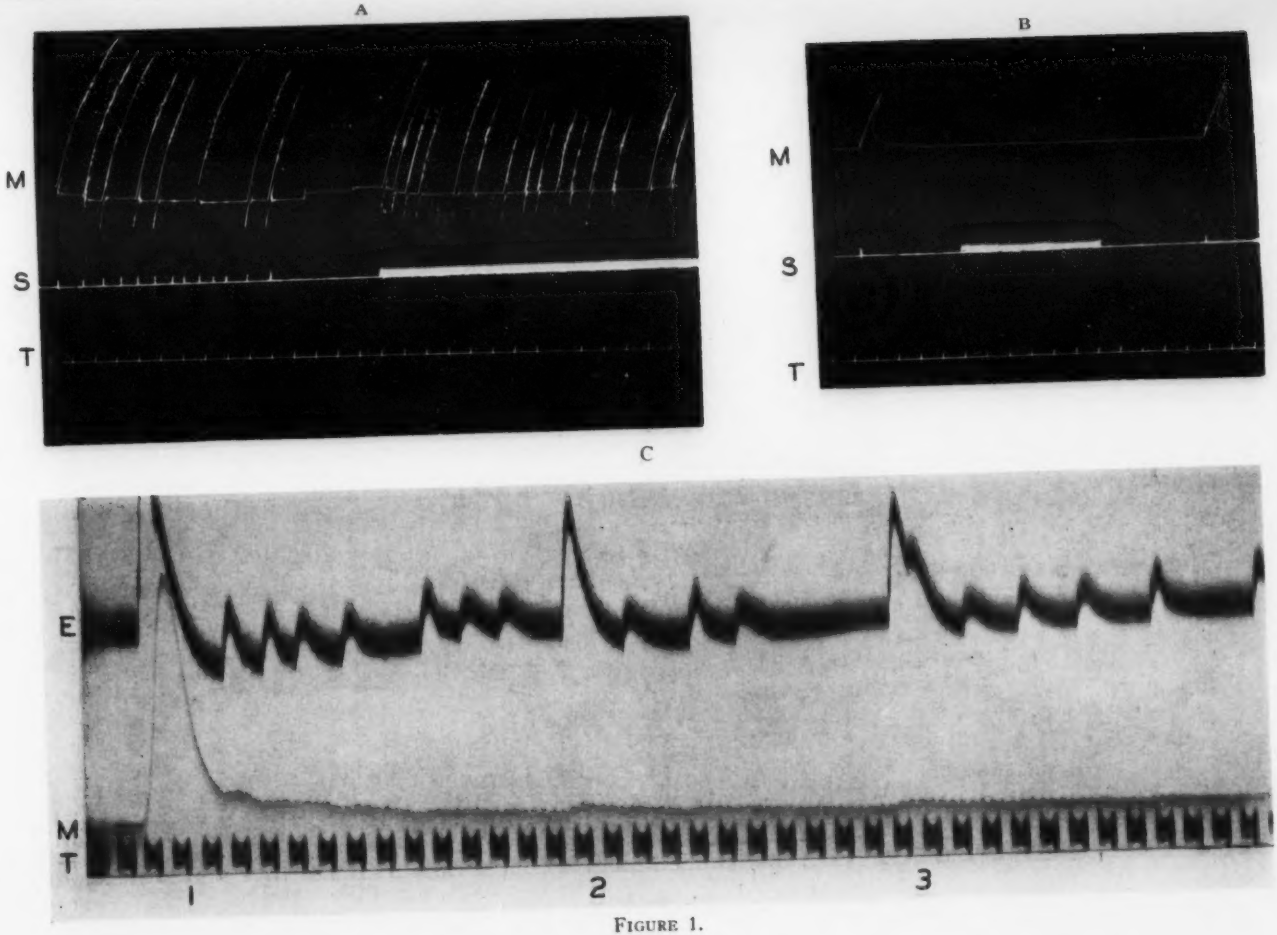

FIGURE 1.

When the fast and the slow systems were stimulated separately the twitch contraction of the closer could be elicited at a time when faradic stimulation of the slow did not result in any visible effect. This might be the result of a selective blocking of the smaller slow fiber before the larger fast one, as was shown by Escobar (1937) to be characteristic for the narcotic block in vertebrate preparations. The difference in the diameters of the "thick" fast closer fiber and the "thin" slow closer axon (58μ and 38μ respectively, see van Harreveld and Wiersma, 1936), however, is hardly great enough to warrant explanation of the early failure of the slow system on this basis. It is a well established fact that in the early stages of narcosis nerve

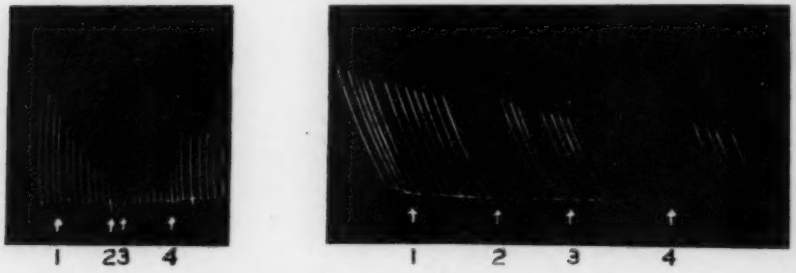

FIGURE 2. Mechanogram of the twitch closer contraction of *Cambarus clarkii* after treatment with procaine. A.—At 1, ½ ml. 0.1 per cent solution of procaine was injected into the claw. At 2, 3, and 4 the claw was washed with van Harreveld solution. B.—The nerve was treated with a 1 per cent procaine solution in a narcotic chamber at 1 and 3. The chamber was emptied and refilled with van Harreveld solution at 2 and 4. Single induction break shocks were given every 30 seconds in A; every minute in B. Note that in A the contraction disappears and reappears gradually, in B, all at once.

fibers exhibit a lengthening of the refractory period (Wedensky, 1904). Because of the dependence of the slow contractions upon facilitation (repetitive stimulation), lengthening of the refractory period would make it impossible for the impulses to follow each other at a high enough frequency to produce a contraction. This seems the more likely explanation for the failure of the slow system before the fast system, especially since in this state faradic stimulation of the fast shows a smaller contraction than the normal one. Drugs producing this anaesthetizing effect when injected into the claw are nupercaine (0.03%), procaine (0.1%), diothane (0.4%), amphetamine (1%), and atropine (1%). The concentrations indicated produced failure in the slow system within five minutes.

Drugs increasing tendency to repeated discharge. Stimulation of crustacean nerves with strong stimuli commonly results in repeated discharge of the nerve fibers. This tendency can be so intensified by treating the preparation with appropriate drugs that a single impulse set up in a normal portion of the nerve fiber, upon reaching the poisoned portion, causes a series of discharges to be initiated at the drugged region. A volley of muscle action potentials is recorded upon stimulation of the nerve with single shocks of weak intensity. Drugs producing this effect are physostigmine, 933F, hydrastinine, pyrethrum, veratrine, and brucine. Veratrine and pyrethrum are effective in much lower concentrations than the others. Injection of these drugs initiates spontaneous contraction in the claw; the larger the dosage the more pronounced is this contraction, and the longer is its duration. These spontaneous contractions are especially pronounced with physostigmine and pyrethrum.

With the electrodes on an undrugged part of the nerve, the concentration of the drugs required to produce repetitive discharges in the slow fiber in response to single shock stimulation is lower than that needed to produce this effect in the fast system. The duration of the burst of repetitive impulses is longer in the slow system than in the fast. In consequence, it has been possible to obtain contractions of the slow type on stimulation of the slow fiber with single weak induction shocks (Figure 3, B 4 and B 5). Repetition of discharge in the fast system results in an increase in the height of the contraction, which is clearly the result of summation of a limited number of impulses following each other within a very short time interval. When 933F was used, for example, a direct relation was observed to exist between the number of action potential spikes in each burst and the magnitude of the contraction. Only with very strong concentrations does a more prolonged fast contraction take place. Pyrethrum [4] (0.02%), 933F (0.1%), and brucine (2.5%) produced only these effects of repetitive discharge.

Hydrastinine presents a somewhat more complex picture. In a concentration of 1 per cent it shows, in the slow systems, essentially the same picture with regard to the number of muscle action potential spikes, thus single impulses will cause contractions. These are, how-

[4] Pyrethrum was kindly supplied by the United States Department of Agriculture in the form of concentrates of Pyrethrin I and Pyrethrin II. The Pyrethrin I concentrate consisted of Pyrethrin I, 57.0 per cent; Pyrethrin II, 13.7 per cent; unknown composition, 29.3 per cent. The Pyrethrin II concentrate consisted of Pyrethrin I, 5.2 per cent; Pyrethrin II, 74.2 per cent; unknown composition, 20.6 per cent. Dilution was made with alcohol to 1 mg./100 ml. This was further diluted with van Harreveld solution to the desired concentration.

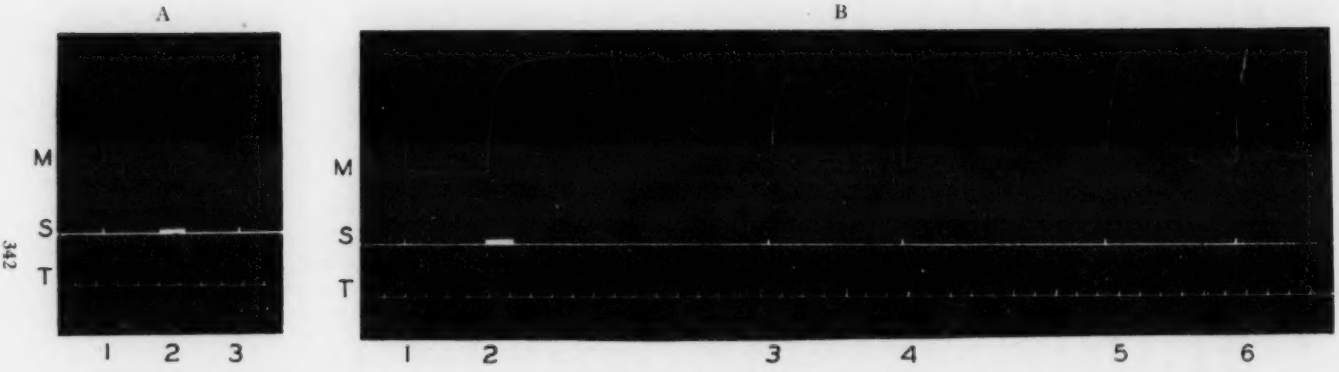

FIGURE 3. Mechanogram of the closer muscle of *Cambarus clarkii* after treating the claw with a 0.001 per cent solution of veratrine. A.—1—normal fast contraction, 2—normal slow contraction, 3—normal fast contraction. B.—1—fast contraction 6 minutes after injection, 2—slow contraction after 6 minutes, 3—fast contraction immediately following 2, 4—contraction following stimulation of slow fiber with single induction shock 7 minutes after injection, 5—slow fiber stimulated with single shock, 6—fast fiber stimulated with single shock 8 minutes after injection. Note that in B-2 the contraction continued for some time after stimulation ceased, and compare this with B-4 in which the response is to a single stimulus. T = 1 second.

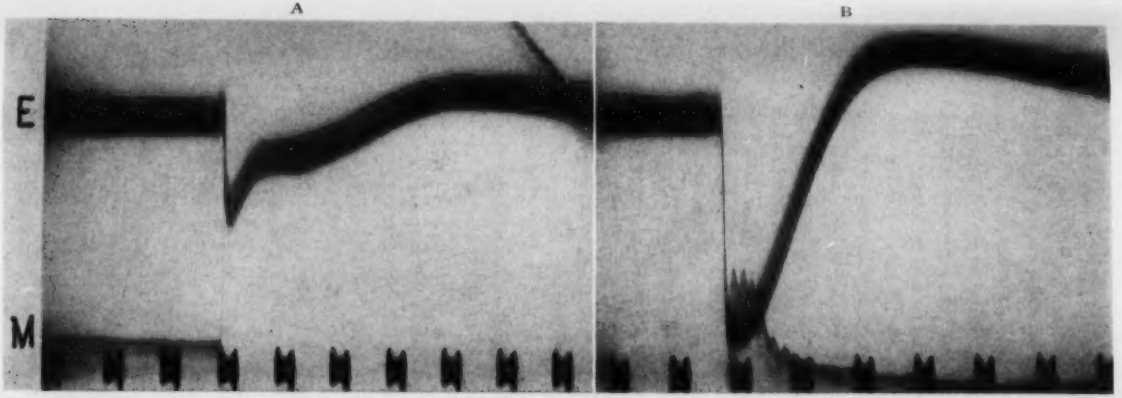

FIGURE 4. Electro- and mechanograms of the fast closer contraction of *Cambarus clarkii*. A.—before and B.—after treatment with 1 per cent hydrastinine hydrochloride. Note the increased size of the muscle action potential and at the same time the diminished size of the mechanical contraction. T = 0.1 second.

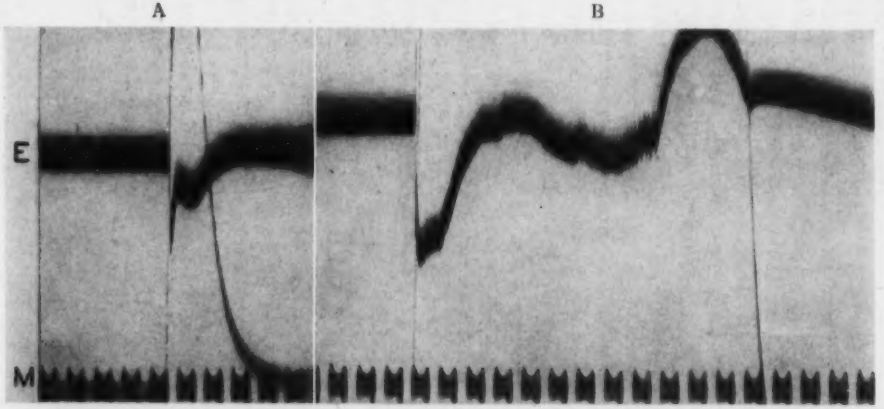

FIGURE 5. Electro- and mechanograms of closer muscle of *Cambarus clarkii* on stimulation of the fast fiber with single induction shocks. A.—normal preparation, B.—same preparation following injection of the claw with a 0.001 per cent solution of veratrine. T = 0.1 second.

ever, of a smaller magnitude than would be expected from a 933F treated preparation. In chamber experiments the fast system shows a higher contraction which is associated with repeated discharge of the nerve fiber, but when hydrastinine is injected into the claw, the mechanical contraction of the fast system diminishes in height, whereas the action potentials show a very pronounced increase in amplitude (Figure 4). Hydrastinine, therefore, appears to exert opposite effects on the muscle action potential and on the mechanical contraction; its effect on the nerve is clearly limited to setting up repeated discharges.

The action of veratrine is due, for the most part, to the initiation of repetitive discharge, but shows, in addition, considerable complication. When the whole nerve is treated in a narcotic chamber there ensues on a single stimulus a twitch of an increased height, sometimes followed by a secondary slow-rising top (Figure 3, B 3). When the slow fiber is removed, however, the contractions show only an increase in the size of the twitch, indicating that the secondary top in the first instance is due to a repetitive outburst in the slow fiber. Upon injection of the claw, single shocks to the slow fiber give rise to more or less prolonged contractions, and faradic stimulation for a short period may be followed by a markedly prolonged slow contraction (Figure 3, B 2). Stimulation of the isolated fast fiber following injection of the claw, however, not only produces contractions of a larger size, but, in addition, the secondary slow-rising top again becomes apparent. Under these circumstances the muscle action potentials show an initial high-topped spike with evidence of repetition, followed by a more or less prolonged burst of much smaller rhythmical action potentials diminishing in frequency, growing in size, and clearly accompanying the second top of the contraction (Figure 5). After the claw has been exposed to the drug for some time, the size of the fast action potentials again diminishes, as does the size of the action potentials of the slow closer contraction and of the opener. The lengthening of the refractory period by veratrine has already been mentioned.

Drugs without apparent effect. A number of substances had little or no effect on the peripheral system in the crayfish, even in high concentrations. These include choline (0.2%), the choline derivatives including mecholyl, doryl, and acetylcholine (0.1%), epinephrine (0.2%), digitalin (1%), nicotine (1%), caffeine (1%), curare (1%), muscarine (0.5%), pilocarpine (1%), and strychnine (0.2%). When injected into the whole animal, however, epinephrine (0.1 mg./g.), curare (0.3 mg./g.), strychnine (0.5 mg./g.), and the tetramethylammonium salts (0.1 mg./g.) had a marked depressive influence on

reflex excitability. Mecholyl (2.5 mg./g.), doryl (0.5 mg./g.), pilocarpine (2 mg./g.), the trimethylammonium salts (0.1 mg./g.), and acetylcholine (1 mg./g.) showed a more or less marked excitatory state as shown by reflex activity. Several of the others, e.g., nicotine (0.01 mg./g.) showed first an increased reflex excitability, but later a depressive effect became evident. These influences indicate an apparent dissimilarity in response between the central and peripheral nervous systems.

Inhibition

As has been pointed out, the experiments on inhibition have made use of the opener preparation in which only a single motor axon and an inhibitory fiber are involved. The drugs which had no effect on excitation were likewise found to have no effect on inhibition. The local anaesthetics and those drugs which lengthened the refractory period had no effect in concentrations which did not depress the contraction, and in stronger concentrations no especial effects were discernible.

The drugs which produce repeated discharges in the nerve fibers produce several interesting effects on the inhibitory mechanisms which can be attributed to this tendency. Because one or both of the fibers may fire repetitively, give after-discharges, or fire spontaneously without any stimulation, the results of stimulation become quite unpredictable, and analysis of them is often not possible. In certain cases, however, analysis can be made with some certainty. The following phenomena have been observed:

After-discharge of the inhibitor is found when, on simultaneous stimulation of the excitor and inhibitor fibers, a sudden stopping of inhibitory stimulation is accompanied by a prolongation of the inhibitory effect.

Both repetitive firing and after-discharge of the excitor have been encountered. The former condition has been observed under certain circumstances quite free from any parallel activity in the inhibitor. Stimulation of both excitor and inhibitor at a frequency of 30/sec. was accompanied, as shown by the muscle action potentials, by three excitatory impulses per stimulus. The mechanical contraction, under these circumstances, was only partially suppressed. This is not surprising since the *frequency of inhibition/frequency of excitation* ratio for complete inhibition in this system is 0.40, two inhibitory impulses being able to suppress 5 excitatory ones. Stimulation of the inhibitor at a frequency of 90/sec. resulted, as expected, in complete inhibition. It is obvious, therefore, that repetitive discharge was limited to the excitor in this case.

An interesting demonstration of the after-discharge of the excitor was seen on releasing inhibition shortly after stimulation of the excitor was discontinued. The resulting contraction could only be due to such an after-discharge.

Combined repetitive activity or after-discharge of both inhibitor and excitor would result in a situation so nearly normal that no clear-cut differences could be observed.

In several instances treatment with hydrastinine (1%) was accompanied by a state in which faradic stimulation of the inhibitor resulted in an enhancement of excitation, or even in contraction of the opener muscle in the absence of stimulation of the excitor (Figure 6). This

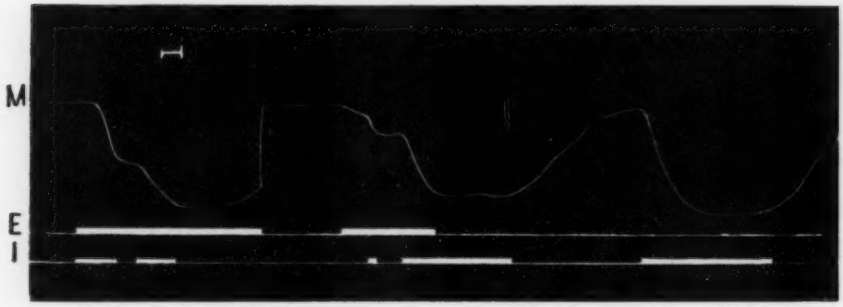

FIGURE 6. Mechanogram of the opener muscle of *Cambarus clarkii* after treatment with hydrastinine hydrochloride, showing reversal of inhibitor. The excitor and inhibitor were isolated so as to prevent stimulation by current escape. T = 1 second, E = excitatory stimulation, I = inhibitory stimulation.

effect was also obtained following injection of pyrethrum (0.02%) into the claw. It should be pointed out that due care was taken in each case to avoid stimulation of the other fibers by current escape. An explanation of this interesting phenomenon will be given in the discussion.

Supplementary inhibition. The presence of an independent secondary inhibitory process (supplementary inhibition) resulting in a depression of the muscle action potentials has made it interesting to investigate the effects of the drugs on this process. Supplementary inhibition occurs only when the time of arrival at the muscle of the inhibitory impulse just precedes that of the excitatory impulse within a very narrow time interval. When yohimbine was used it was found that with concentrations which were just high enough to ultimately produce the characteristic block of excitation, supplementary inhibi-

tion was present, apparently unchanged, up to the very moment of the excitatory block. This was also the case with the local anaesthetics. Hydrastinine, veratrine, and pyrethrum showed no selective effect on this process. These three latter substances, however, by virtue of their tendency to set up multiple discharges in the nerve upon stimulation, produce a state in which many of the excitatory impulses are firing at times which make their arrival at the muscle fibers with respect to the arrival of the inhibitory impulses fall outside the narrow time limits necessary to produce depression of the muscle action potentials. By using a cathode ray oscillograph with the sweep synchronized with the stimulation frequency, it was possible, in several instances, to perceive, instead of one, two or more action potentials in each sweep. Only the first of these showed any reduction during supplementary inhibition.

Pyrethrum (0.0001%) greatly increased the excitability of the axons, giving rise to a state in which almost any degree of activity could be obtained. In the intervals between spontaneous discharges, reduction of the muscle action potentials during inhibition was still possible. Veratrine in low concentrations (0.001%) resulted in a situation quite similar to that following injection of hydrastinine; the first action potential was reduced, the repetitive ones were unchanged. It appears, therefore, that these substances have little effect on the mechanism of supplementary inhibition.

Discussion

In view of the fact that the innervation of a muscle fiber in the decapod crustacea presents a picture so completely different from that of vertebrate skeletal muscle, it is not unexpected that responses of such a nerve-muscle preparation to drugs should be, in general, quite different. Straub (1900) and Katz (1936) have shown that curare, e.g., which is known to block the transmission mechanism at the end-organ in vertebrate preparations, has no noticeable effect on the crustacean system. Our results further illustrate the fundamental differences in the action of drugs on vertebrate and crustacean preparations.

The hope that by using a number of drugs it would be possible to study more clearly the mechanism involved in the transmission of the nerve impulse to the contractile mechanism in the crustacea has not been realized. Katz (1936) reported a "curarization" effect on crustacean muscle on treating the preparation with an excess of Mg^{++}, but it was certainly not well borne out by Waterman (1941), nor has it been possible in our laboratory to reproduce this effect. In fact

it has not been possible to observe any effects which could be limited to the transmission mechanism between nerve and muscle. A large majority of the substances used have clearly exerted their effects on the nerve itself, the response being the same or very similar whether the drug was injected into the muscle or whether the nerve was treated in a narcotic chamber. Both hydrastinine and veratrine show, besides an effect clearly limited to the nerve fiber, an effect which seems to be located somewhere between the nerve impulse and the contractile

TABLE II

Classification of drugs as to their action on Crustacean peripheral nerve-muscle systems

Group	Local anaesthetics	Lengthened refractory period	Increased tendency to repeated discharge	No effect (concentrations up to 2 per cent)
Drug	Procaine* Diothane* Nupercaine† Amphetamine Atropine	Yohimbine‡ Veratrine‡	Veratrine‡ Pyrethrum† Physostigmine† 933F* Hydrastinine Brucine	Choline Acetylcholine Mecholyl Doryl Muscarine Curare Strychnine Trimethylammonium salts Tetramethylammonium salts Pilocarpine Digitalin Epinephrine Nicotine Caffeine Rotenone

* Effective in concentrations between 1 and 0.1 per cent.
† Effective in concentrations between 0.1 and 0.01 per cent.
‡ Effective in concentrations below 0.01 per cent.

mechanism. The change brought about on the nerve impulses, however, prevents satisfactory analysis of this additional effect.

In many cases noticeable effects were obtained only by using strong concentrations of substances which, in mammals, are known to work in very low concentrations. One hesitates, therefore, to consider the action of substances like prostigmine, which gave little effect in concentrations of less than 2 per cent as specific. Certain of the results, however, can be discussed with a reasonable degree of confidence (Table II).

Aside from the narcotizing effect of the local anaesthetics, the effects of the drugs on the nerve seem to be of two types; either the

refractory period is so lengthened that the nerve cannot carry impulses at an effective frequency, or the excitability is so increased that multiple discharges are set up. Either of these effects provides interesting relationships to the problems involved in the "twitch" and "slow" contractions of crustacean muscles. The characteristic need for facilitation in the slow contractions is emphasized by the former type of response, i.e., lengthening the refractory period to such an extent that facilitation becomes impossible, whereupon no slow contraction is obtainable. On the other hand, those drugs which increase the excitability and thereby give rise to multiple discharges on single shocks, show contractions of the slow type on stimulation with single weak shocks which normally produce no response whatsoever.

It should be pointed out that the fundamental mechanisms involved in inhibition were apparently little influenced by even the most effective drugs used in this investigation. Drugs which lengthen the refractory period of the nerve cannot be easily investigated as to their effect on peripheral inhibition, since their effect on the excitatory mechanism in "slow" systems usually results in a complete excitatory failure. It is probable, however, that they exert an effect on the inhibitory axon identical with that on the motor fiber. Reduction of the muscle action potentials during "supplementary" inhibition was found to apply only to the action potential set up by the motor stimulus; subsequently occurring repetitive discharges, being asynchronous with inhibitory ones, showed no such depression. Simple inhibition, at least qualitatively, appeared to be quite normal.

The "reversal" effects in which stimulation of the inhibitor results in contraction, or stimulation of the excitor results in inhibition, need a more extended discussion, especially in the light of Segaar's (1929) claim that inhibitory stimulation at times becomes excitatory and vice versa. He attributed this to an inherent ability of the axons to produce reciprocal effects, i.e., the inhibitory axon actually becomes excitatory under certain influences, and vice versa. The present investigation has provided some pertinent information concerning this type of behavior, which makes explanation of the phenomena possible without having to assume any alteration of the inherent specificity of the nerve fibers. The reversal effects are not normally encountered, and are only produced with great difficulty. They are rarely, if ever, encountered together in the same preparation.

In the crayfish opener system, the motor axon is much more prone to repeated discharge than is the inhibitor. This is seen on cutting the leg from the intact animal, whereupon, following a short-lasting initial closing, a prolonged opener contraction is almost always present.

Stimulation for this contraction is a spontaneous volley arising at the severed end of the motor axon. Were the inhibitor equally prone to repetitive discharge, the contraction would be suppressed by the spontaneous impulses arising from the cut end of the inhibitory fiber. Observations in this laboratory have shown that of all the nerve fibers in the claw, the opener axon shows by far the most prolonged after-discharge following transection of the nerve fibers. It is not surprising, then, that certain drugs (veratrine, pyrethrum, etc.) should set up repetitive discharges in the motor axon to a greater extent than in the inhibitory fiber, nor is it difficult to conceive that under these circumstances a spontaneous motor discharge is set up at a frequency such that the nerve fiber is barely able to follow. Additional stimulation of the motor axon would then result in a Wedensky-block of the opener contraction. This concept is further supported by the complete absence of muscle action potentials, showing that the excitatory fiber is quiescent. If the absence of contraction were due to inhibition, action potentials of at least 25 per cent of the normal height should be present.

The other reversal effect in which stimulation of the inhibitor produces a contraction or an enhancement of the one set up by excitation, however, cannot be explained on this basis. The total absence of muscle action potentials shows that the excitor and inhibitor were not both firing spontaneously and thus suppressing the contraction until released by a Wedensky-block of the inhibitor. It is apparent, therefore, that some other mechanism is involved. It seems likely that the nerve fibers (probably in the smaller branches) become so sensitive under the influence of the drug that passage of an impulse along the inhibitory fiber produces sufficient polarization to excite the hypersensitive adjacent motor axon. In these motor branches each such stimulus sets up repeated discharges, raising the excitatory frequency above that which the inhibitory frequency can suppress. Such cross excitations have been reported (Jasper and Monnier, 1938) and the interaction between adjacent fibers has been demonstrated and studied by Katz and Schmitt (1940, 1942).

Summary

The effects of a wide selection of drugs on the peripheral nerve-muscle preparations of the cheliped of the crayfish, *Cambarus clarkii*, were studied.

Of all drugs used, the local anaesthetics were the only ones which, as a group, showed the customary vertebrate effects on crustacean preparations.

In general, the effects of the drugs used in the investigation were limited to changes in the excitability of the nerve fiber. These effects were of two types; either the refractory period of the nerve was markedly lengthened, or the nerve fiber became so hypersensitive that excitatory stimuli set up multiple discharges in the nerve.

The effects of the drugs on peripheral inhibition were studied, with the conclusion that the substances exert little effect on the mechanisms involved in these phenomena, especially on those responsible for supplementary inhibition.

An investigation was made into certain "reversal" effects in which stimulation of the inhibitory axon showed an excitation, and in which stimulation of the excitatory axon showed an inhibitory effect. Of these, the former is ascribed to stimulation of the adjacent hypersensitive motor fiber upon stimulation of the inhibitor; the latter, to a Wedensky-block.

The central effects appear to be quite different from the effects on the peripheral systems.

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OXYGEN CONSUMPTION OF FOX SPERM

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Measurements of oxygen consumption have been made on human spermatozoa (Shettles, 1940; MacLeod, 1941) and on those of some domestic and laboratory animals (Ivanow, 1931; Redenz, 1933; Windstosser, 1935; Comstock, 1939; Lardy and Phillips, 1941; Henle and Zittle, 1942). The determinations have been carried out on suspensions of epididymal sperm derived from excised gonads and suspensions of seminal sperm obtained by emission.

Natural and technical difficulties attending the measurement of respiration have hitherto required certain preparatory changes to be made in the normal sperm suspensions before the respiratory measurements were made. It has often been necessary to dilute densely packed samples of epididymal sperm suspensions, and most investigators have found it expedient to remove the sperm altogether from the normal medium by centrifugation and to resuspend the sperm in Ringer-glucose or salt solution (MacLeod, 1941; Henle and Zittle, 1942).

In suspensions in which the count is low, it has often been necessary to concentrate the sperm by centrifugation in order to secure sufficient respiring material to give a measurable oxygen uptake. Frequently samples have been pooled for determinations (Henle and Zittle, 1942). This procedure supplies much material for study but cancels individual variation. It was found necessary to use one ml. of suspension in the Warburg apparatus (MacLeod, 1941), but samples of that size are not easily obtained from small animals. A further important drawback in many investigations of the respiration of sperm is the considerable delay that is allowed to lapse between the time the sperm are collected and the determinations made.

By the use of the microrespirometer of Scholander (1942), it is possible to eliminate several of the difficulties encountered in previous measurements. The measurements were made on the semen from silver foxes, for in the short breeding season of these animals objective criteria of the viability of sperm are significant. Very small quantities of ejaculate, containing relatively few sperm, could be measured,

* Agent, Fish and Wildlife Service, United States Department of the Interior.

and the sperm were studied in the seminal fluid, essentially as they are under natural conditions. Furthermore, only a short period (about ten minutes) intervened between the time the sperm were collected and the respiratory measurements begun. A continuous record of oxygen consumption could be obtained over a two-hour period.

Methods and Materials

Apparatus

The volumetric microrespirometer described by Scholander (l.c. Figure 3) was used in this investigation. It was necessary to change the apparatus in several respects to adapt it to this problem (Figure 1).

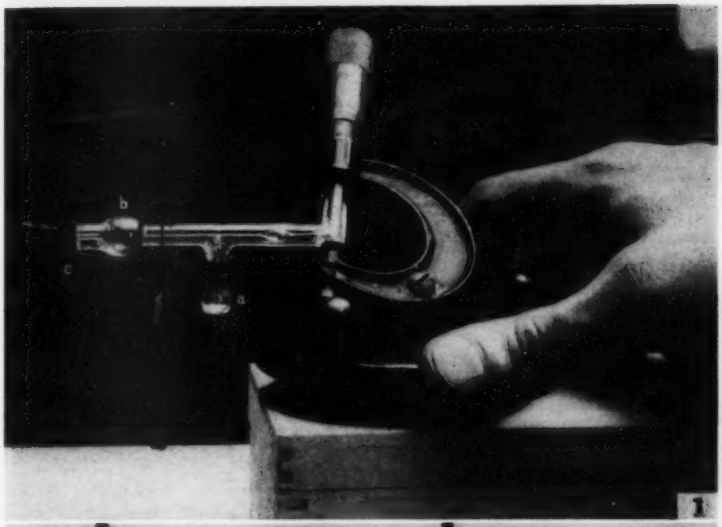
A bulb-shaped respiratory chamber, *a*, of about 0.25 cc. total capacity, replaces the original straight-sided chamber. This vessel offers a larger surface on which to place the sperm suspension, and at the same time is easier to manipulate, without decreasing the sensitivity of the instrument.

The opening of the capillary tube into the compensatory bulb, *b*, is sealed with a piece of rubber held in place by a brass clamp, *c*. This seal avoids the use of grease which often and especially when wet oxidizes sufficiently to indicate a considerable oxygen consumption. The blank obtained when grease is used is too large to be disregarded.

Determinations on mammalian sperm require a water bath kept at 37°–38° C. (Figure 2). The thermobarometer does not require closer control of temperature than to within one degree. Two baths were used, one within the other, to prevent sudden temperature changes around the apparatus. The entire instrument, including the micrometer, was immersed in the inner bath.

Sensitivity

When the apparatus was set up without respiring material, the volume change was not greater than 0.01 cu. mm. an hour. This figure represents the limit of sensitivity and stability. Under these conditions there is practically no blank value. It was found that measurements of oxygen consumption could be made on suspensions containing as few as 500,000 sperm. With fewer sperm the values were open to question. When suspensions containing approximately 100,000 sperm were tested, the oxygen consumption could barely be detected. Satisfactory determinations of sperm respiration were obtained with 0.02 cc. samples in which the concentration of sperm was as low as 30,000,000/cc. In the fox the volume of the ejaculate, obtained by the electrical stimulator, varies between 0.5 and 3.0 cc., of



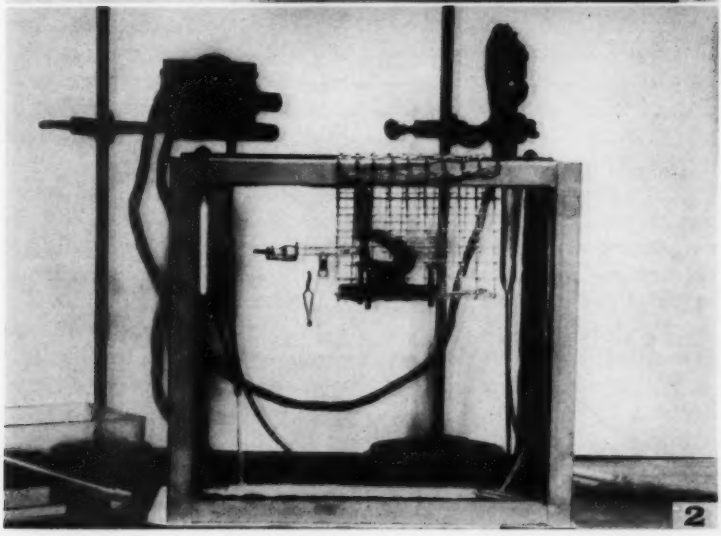


FIGURE 1. The microrespirometer showing *a*, the respiratory chamber; *b*, compensatory bulb; *c*, brass clamp which holds rubber seal in position.

FIGURE 2. The microrespirometer in position in outer water bath with heater and thermoregulator; inner bath removed.

which one-half to one-fourth is sperm fraction, the remainder being relatively sperm-free prostatic and preputial secretions. The sperm concentration normally lies between 25–240 million/cc.

Procedure

The spermatozoa of the silver fox, *Vulpes fulva*, were obtained by means of an electrical stimulator (Benham and Enders, 1941). The ejaculate was collected in a glass-tipped syringe and immediately transferred to small centrifuge tubes. Mild centrifugation by means of a hand centrifuge (60 r.p.m. for 25 seconds) was sufficient to separate the sperm from tissue debris and preputial oil globules. The concentration of the sperm in the middle of the suspension remained unaltered.

Approximately 20 cu. mm. of sperm suspension were introduced into the bottom of the respiratory chamber, which together with the rest of the respirometer, had been kept in an oven at about 37° C. Potassium hydroxide (0.9 per cent) was added to the platinum wire loop suspended from the T-tube so as to project into the respiratory chamber. The chamber, containing the sperm, was then placed firmly in position, after which a drop of 2 per cent Tergitol was introduced into the capillary tube through the compensatory vessel, and the tube closed with the rubber seal. The apparatus was immersed in the water bath, care being taken to compensate for the initial drift of the Tergitol drop owing to temperature changes. About ten minutes elapsed between seminal emission and the beginning of the respiratory determinations.

As oxygen was consumed by the respiring material, and carbon dioxide absorbed, the volume of the gas phase in the animal chamber and the capillary tubes became smaller as indicated by the drift of the meniscus in the tube. By screwing in the plunger of the micrometer this drift could be compensated and the meniscus returned to the same position in the capillary tube. Oxygen consumption was read in micrometer scale divisions and these converted into units of volume.

The oxygen consumed in one hour was on the order of one cu. mm., less than 2 per cent of the total oxygen in the system at the beginning of a determination. No oxygen was added to the reservoir during the course of the measurements.

The sperm suspension was spread in a thin film, about 0.5 mm., in thickness, on the bottom of the respiratory chamber. Diffusion was believed to be sufficiently rapid so that agitation was not necessary.

After the respiratory measurements were made, the sample was transferred to a graduated tube and diluted 500 times. With a blood-counting pipette a known volume of the sample (one part in

11,000) was placed on a hemocytometer slide, and the number of sperm determined. Duplicate counts with an average error of less than 10 per cent were obtained.

RESULTS

Oxygen consumption of fox sperm suspensions

The initial uptake of oxygen by the sperm suspension frequently is very high, but after a stabilization period, from two to 15 minutes,

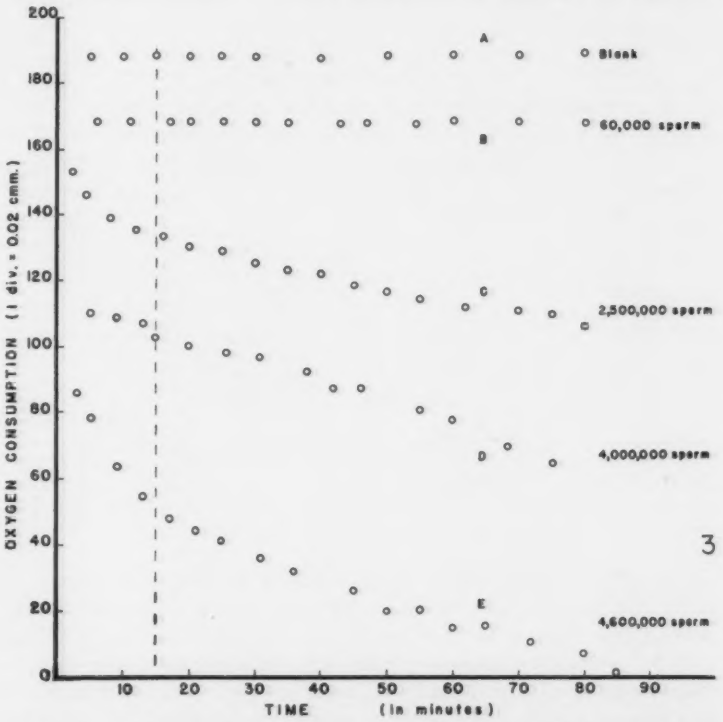


FIGURE 3. Oxygen consumption of fox seminal sperm during first 1½ hours after emission. *A*, a blank run with no sperm in respirometer. *B*, a run on a sample with very low sperm count (5,000,000/cc.). *C*, *D*, and *E* are determinations on samples of 2,500,000; 4,000,000 and 4,600,000 sperm, respectively. First 15 minutes a stabilization period.

continues steadily at a lower rate for one to two hours (Figure 3, E). The initial high oxygen consumption may be attributed to adjustment to temperature changes during transfer. But the regularity of the

initial increase, rather than a random increase or decrease, in oxygen consumption during the first few minutes seems to suggest a natural rapid initial respiration which might be brought about by the mixing of the sperm with prostatic secretions just prior to ejaculation. The absence of preliminary adjustment period in the blank would indicate that the apparent initial high oxygen uptake is characteristic of the sperm suspension and not of the apparatus.

Determinations of the oxygen consumption of sperm from six foxes are presented in Table I, expressed in cu. mm. oxygen consumed

TABLE I

Oxygen Consumption of Fox Sperm

Measurements based on first hour after stabilization period

Fox	cu. mm./10^8 sperm/hr.	Number of sperm in sample (0.02 cc.)	Remarks *
915 M	26.6	600,000	Uncentrifuged
191 A	26.7	3,500,000	Uncentrifuged
35 B	27.3	4,400,000	Uncentrifuged
35 B	19.2	4,600,000	Uncentrifuged
255 B	18.0	1,670,000	Centrifuged
161 B	20.8	2,500,000	Centrifuged
255 B	19.2	3,750,000	Centrifuged
255 B	17.5	4,000,000	Centrifuged
35 B	14.7	4,070,000	Centrifuged
35 B	5.4	10,000,000	Centrifuged; sperm motility sluggish, many free tails (late in season)

* A hand centrifuge (60 r.p.m. for 25 seconds) was sufficient to separate the sperm fraction from the oily secretation and the tissue debris.

per 100,000,000 sperm per hour (ZO_2 value of Redenz and of Henle and Zittle). Excluding the last determination, made on a sample with many immotile and broken sperm, the values lie between 19.2 and 27.3 for uncentrifuged suspensions, and between 14.7 and 20.8 for suspensions from which epithelial cellular debris was centrifuged off. The measurements cover the first hour after the stabilization period.

The amount of oxygen consumed, plotted against time, is shown in Figure 3, for three determinations. Included in the graph are a blank run (*A*) and a determination on a suspension with too few (60,000) sperm to measure any oxygen consumption (*B*). As the concentration of sperm increases, the slope of the curve becomes steeper.

An appreciable variation was found in determinations made on sperm from the same fox on successive days. A diminished respiratory

rate seemed to be correlated with a decrease in reproductive activity as indicated by the production of fewer sperm, a decrease in motility of the sperm, and a reduction in volume of ejaculate.

When the ejaculate was secured during the middle of the breeding period, the oxygen consumption of the sperm suspensions was proportional to the number of sperm present. Figure 4, based on the data in Table I, shows that with a threefold increase in sperm concentration

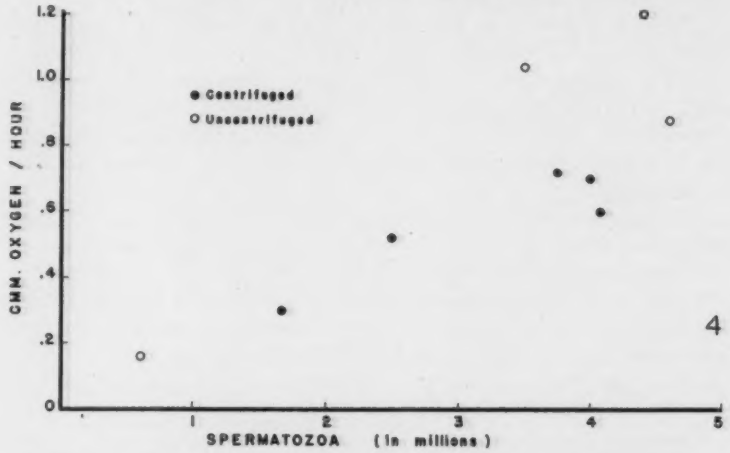


FIGURE 4. Rates of oxygen consumption of sperm suspensions of different concentrations during first hour after stabilization period. The oxygen consumed is proportional to the number of sperm present. The high values of two uncentrifuged samples due to contamination by cellular debris. All determinations made on 0.02 cc. samples.

among the centrifuged samples and an eightfold increase among uncentrifuged samples the increased respiration was proportional to the increased concentration.

Oxygen consumption of components of semen

Samples of semen frequently showed such unusually high oxygen consumption as to suggest the presence of respiring material other than sperm. The seminal fluid was centrifuged at low speed which was sufficient to isolate for measurement three fractions: (1) the lighter oily secretions derived mainly from the preputial glands, (2) the middle sperm-containing portion, (3) and the relatively sperm-free sediment consisting of dead and cornified cells and tissue debris, which in part was traced back to the epididymis in origin.

(1) The lipoidal secretions from the preputial glands had no detectable oxygen uptake.

(2) The middle portion, consisting of sperm and prostatic secretions, consumes oxygen. If the sperm were very few in number (5,000,000/cc. or less), or lacking altogether, as frequently is the case in animals at the close of the breeding season, the prostatic secretions showed no oxygen uptake. Samples with immotile and apparently dead sperm did not consume oxygen.

(3) The sediment in the bottom fraction respires at a high rate. This material may convey to entire semen a large oxygen consumption regardless of the number of sperm present. This sediment was traced back in part to the epididymis and it is probably comparable to the "epididymal secretion" of Henle and Zittle (1942) which showed a slight oxygen consumption and which augmented sperm respiration considerably.

Urine was tested to determine whether it might contribute anything as a contaminant to the total oxygen consumption of semen. No respiration was detected.

The point to be emphasized in this connection is that no oxygen consumption can be detected for semen without sperm, so long as the secretions are not contaminated by epithelial cells and debris.

Oxygen consumption of dog sperm suspensions

Several determinations were made on the ejaculates of a dog. The average oxygen consumption found of 21 cu. mm./10^8 sperm/hour is about the same as the fox. This is significant in the light of the fact that the seminal sperm of the dog are viable much longer than are those of the fox. Fox sperm did not retain motility longer than three hours after ejaculation; dog sperm were still motile 24 hours after ejaculation, although they were allowed to cool to room temperature (ca. 20° C.). The seminal fluid of the dog contains a negligible amount of tissue debris, and reliable results may be obtained on seminal sperm without centrifugation.

Discussion

A comparison of the respiratory determinations of fox and dog sperm with measurements of other investigators on other mammalian sperm is summarized in Table II. The environmental modifications to which most samples were subjected before and during observation may easily be responsible for some of the variations. The low rate of aerobic respiration of human seminal sperm after the first half-hour has been emphasized by MacLeod, who found practically no uptake

of oxygen when the sperm were suspended in Ringer or Ringer-glucose solution. However, when p-phenylene diamine (M/50) was added to the substrate the oxygen consumption was comparable to that for fox sperm (MacLeod, 1942).

The oxygen consumption of sperm may be expressed in terms of cc./gm./hr., although the calculation is only an approximation, based

TABLE II

Comparison of oxygen consumption determinations of suspensions of mammalian spermatozoa

Source	cu. mm./10^8 sperm/hr.	Authority
Rat, Guinea pig, Bull epididymis	0–38	Windstosser, 1935
Bull epididymis	7–30 (av. 18)	Redenz, 1933
Bull epididymis	11–27 (av. 15)	Henle and Zittle, 1942
Bull semen	7–10 (av. 9)	Henle and Zittle, 1942
Bull semen	8–28 (av. 19)	Lardy and Phillips, 1941
Boar semen	3–12.5*	Winchester and McKenzie, 1941
Human semen	0.3–3 (av. 1.8)	MacLeod, 1941
Human semen	18†	MacLeod, 1942
Human semen	15–17 (av. 16)	Shettles, 1940
Fox semen	14–30 (av. 19)	Bishop
Dog semen	17–26 (av. 21)	Bishop

* Higher values were consistently obtained with lower concentrations of sperm.
† A high oxygen consumption by the human sperm was obtained upon the addition of p-phenylene diamine to the substrate.

on a rough measurement of the volume and the assumption that the specific gravity of the suspension is close to one. For fox sperm the values vary from 1.5 to 3.6, and for the dog between 4.8 and 5.4 cc./gm./hr.

Windstosser (1935) claimed to have demonstrated oxygen consumption by suspensions of immotile epididymal sperm. In the fox no oxygen consumption was detected in samples with immotile sperm, except where attributable to contamination. The possibility is not eliminated that immotile sperm consume oxygen, but if this is the case we were not able to measure it.

SUMMARY

A volumetric microrespirometer was used to measure the oxygen consumption of fox sperm obtained by electrical stimulation. Determinations were made on unmodified semen and on the components of semen during the first 1½–2 hours following ejaculation. After an initial high stabilization period, lasting up to 15 minutes, the oxygen uptake of sperm suspensions averaged 19 cu. mm./10^8 sperm/

hour at 37°–38° C. The oxygen consumption was proportional to the number of sperm present over an eightfold range. Neither sperm-free semen, preputial secretions, prostatic fluids, nor urine consumed oxygen if not contaminated by tissue debris.

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A NOTE ON THE FREEZING-POINTS OF THE URINES OF TWO FRESH-WATER FISHES: THE CATFISH (*AMEIURUS NEBULOSUS*) AND THE SUCKER (*CATOSTOMUS COMMERSONII*)

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In his book, *Osmotic Regulation in Aquatic Animals*, Krogh (1939) comments on the scarcity of available data for the osmotic concentration of the urines of fresh-water fishes. Since such data are apparently not available for two of our common local forms, the catfish (*Ameiurus nebulosus*) and the sucker (*Catostomus commersonii*), we have been interested to make freezing-point determinations of their urines. During the urine collections, incidental observations on rate of flow were also included.

The fish, which were obtained locally, were kept in the laboratory fish tanks, and care was taken to use only fish in good condition. They were given no food during the experimental period. One series of determinations was made during the winter (November through February); the second, shorter series was made in May.

Urine was collected from the catfish through a small cannula tied into the urinary papilla and anchored by a stitch to the body wall. The clamping of a tube attached to the cannula prevented loss of fluid during the collection period; upon releasing the clamp, the urinary bladder could readily be emptied by pressure gently applied to the ventral body wall. In the case of the sucker, a similar cannula was used but the absence of a urinary bladder made necessary the attachment of a rubber balloon for collecting the urine.

By using a specially constructed container, freezing-point determinations could be made on amounts of fluid as small as 4 cc. A Heidenhain thermometer was used, graduated in 1/100° C. intervals, from which readings could be estimated to 2/1000° C. The Δ of the fresh-water medium was found to be .002° C.

Eleven urine samples from 5 catfish and 13 samples from 8 suckers were used for the determinations. The Δ values for the urines, together with the rates of urine flow, are summarized in Tables I and II. Corresponding to the relatively faster rates of flow the urine of the

catfish was extremely dilute, in comparison with the slower flow and more concentrated urine of the sucker. Although the data are not full enough to give definite information as to seasonal variations, they

TABLE I

Urine Flow and Δ of the Urine of the Catfish, Ameiurus nebulosus

Fish No.	Date	Body Wt., gm.	Collection Period, hrs.	Urine Flow, cc. per kgm. B.W. per hour	Δ of Urine, °C.
1	Nov. 1	333	33	2.1	.034
	Nov. 2		14	2.7	.035
	Nov. 8		9	2.5	.030
				Av. 2.4	Av. .033
2	Nov. 22	241	17	1.7	.021
3	Jan. 9	...	...	...	.029
4	May 16	510	4	2.4	.019
	May 16		5.5	3.7	.021
	May 16		2.5	3.9	.023
	May 17*		10	3.0	.022
				Av. 3.25	Av. .021
5	May 17	335	7	3.2	.023
	May 17		4.5	3.6	.019
				Av. 3.4	Av. .021
Averages				(Winter) 2.1 (Spring) 3.3	.025

* Subsequent blood sample gave Δ serum value of .49° C.

suggest for both species a faster rate of urine flow in the spring than in the winter.

DISCUSSION

Our average Δ value of .094° for the urine of the sucker checks closely with Smith's (1932) figure of .09° for the fresh-water eel and rather well with the .07° and .08° which he obtained for the two ganoids, the bowfin *Amia* and the gar pike *Lepidosteus*, respectively. The distinctly lower Δ value of .025° for the catfish urine is doubtless correlated with its faster flow, although our fish were far from attaining the daily output of 300 cc. per kgm. of body weight that Marshall (1934) reports. The relatively fast urine flows in the catfish are of interest from the standpoint of the large glomerular filtering surface in this fish. Marshall and Smith (1930) have shown that the catfish renal

corpuscle is larger than that of many other fresh-water fishes: 99μ as against 60μ in the sucker. Also, studies made by Nash (1931) show that the catfish leads a group of eight fresh-water fishes studied with respect both to glomerular number and glomerular volume per unit

TABLE II

Urine Flow and Δ of the Urine of the Sucker, Catostomus commersonii

Fish No.	Date	Body Wt., gm.	Collection Period, hrs.	Urine Flow, cc. per kgm. B.W. per hour	Δ of Urine, °C.
1	Jan. 17	672	36	0.22	.047
	Jan. 19		25	0.30	.077
				Av. 0.26	Av. .062
2	Feb. 2	895	24	0.26	.069
3	Feb. 2	850	24	(0.44)*	.086
4	Feb. 10	1210	36	0.14	.120
	Feb. 10		8.5	0.54	.092
				Av. 0.34	Av. .106
5	Feb. 10	1125	36	0.27	.181
6	Feb. 20	1007	15	0.35	.046
	Feb. 21		16.5	0.26	.062
	Feb. 21		10.5	0.42	.073
				Av. 0.34	Av. .060
7	May 8	1079	18.5	0.38	.107
	May 8		8	1.6+	.137
				Av. 0.99	Av. .122
8	May 11	778	8.5	1.2+	.064
Averages				(Winter) 0.29 (Spring) 1.10	.094

* Approximate only; omitted from average.

of body surface area. Unfortunately, Nash's figures do not also include values for the sucker.

Seasonal variations related to activity, nutritional status, and breeding should be investigated before the ranges of values for Δ and flow of urine can be definitely stated.

SUMMARY

1. Freezing-point determinations on the urines of five catfish, *Ameiurus nebulosus*, collected in winter and in May, ranged from −.019° C. to −.035° C., with an average of −.025° C.

2. Similar determinations on the urines of eight suckers, *Catostomus commersonii*, showed a range from −.046° C. to −.181° C., with an average of −.094° C.

3. The rate of urine flow was considerably higher in the catfish than in the sucker.

4. The possibility of seasonal variations in urine flow is suggested.

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INDUCTION OF TRIPLOIDY AND HAPLOIDY IN AXOLOTL EGGS BY COLD TREATMENT

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Recent investigations on polyploidy in three species of salamanders have shown that spontaneous deviations in chromosome number occur with surprising frequency among the larvae (Fankhauser, 1941b) and that it is relatively easy to induce triploidy experimentally by refrigerating eggs immediately after fertilization. This treatment presumably suppresses the second maturation division which is normally not completed until about one hour after laying (Fankhauser and Griffiths, 1939; Griffiths, 1941; Fankhauser, 1942).

The effects of the addition of a third chromosome set on development and growth have been studied in detail. Triploidy, as well as higher degrees of polyploidy, do not produce a significant increase in body size in salamander larvae, since the larger size of the individual cells is compensated by a corresponding decrease in cell number (Fankhauser, 1941a). On the other hand, triploidy was found to have a striking effect on sex differentiation; at the time of metamorphosis triploid testes appear normal, while triploid ovaries are much reduced in size and greatly retarded in differentiation (Fankhauser, 1940). Since in other animals triploidy affects the heterogametic sex only, this indicated that the female salamander produces two kinds of gametes, while the male is homogametic.

In view of these interesting and partly unexpected effects of triploidy in salamanders it seemed desirable to extend the work to still other species. Experiments with axolotl eggs had been planned for some time because axolotls may be raised and bred in the laboratory with comparative ease, and because a large amount of information is available regarding the development of the gonads under normal and experimental conditions. It is to be expected that the effects of polyploidy on sex can be analyzed more completely in this species than in those used before in which not even the normal process of sex differentiation had been described. The preliminary experiments to be reported in this paper were prompted by the recent investigations on the sex ratio in the progeny of sex-reversed Amblystoma females,

which produced convincing evidence that the female is normally heterozygous with regard to the sex chromosomes (Humphrey, 1942).

Materials and Methods

Eggs of the white (partial albino) variety of axolotls which are kept and bred in the Department of Anatomy of the University of Buffalo were used exclusively. The experiments were performed in Buffalo, and the embryos developing from the treated eggs, as well as untreated controls, were then shipped to Princeton for identification of the chromosome number of each animal and for the study of their later development.

The eggs to be refrigerated were removed immediately, or within a few minutes, after laying (which coincides with fertilization) and put in a container with ice water at + 1° to + 3° C., for from nine to 24 hours. Following this treatment they were transferred at once to dishes kept at room temperature and allowed to develop.

Shortly after hatching the posterior half of the tail of each larva was amputated and fixed in Bouin's or Navashin's fluid for two to four hours. These tailtips were stained in toto with Harris' acid haemalum and mounted, and the chromosomes counted in mitotic figures in the epidermis of the transparent tailfin.

In white axolotl larvae the fin surrounding the tail is practically free from melanophores which in normally pigmented larvae interfere with the microscopical study. However, when compared with tailtip preparations of larvae of *Triturus viridescens*, those of the axolotl larvae have several disadvantages: (1) many preparations contain very few mitotic figures; in some tailtips they are completely absent; (2) the diploid chromosome number is 28 against 22; (3) the arrangement of the chromosomes at metaphase is less regular and makes counting more difficult; (4) the cells are from 10 to 20 per cent smaller.

Results of Refrigeration Experiments

The results of the refrigeration experiments are summarized in Table I. The mortality among the treated eggs was high. Many eggs either did not cleave or divided abnormally and died at or before gastrulation. About 20 per cent of the eggs developed to hatching larvae, as compared with from 40 to 45 per cent in similar experiments with eggs of *Triturus viridescens*, and with 25 per cent in experiments with *Triturus pyrrhogaster* (Fankhauser, Crotta and Perrot, 1942).

Cytological examination of the tailtip preparations showed that the great majority of the larvae developing from refrigerated eggs—25 of 31, or about 80 per cent—were triploid. The absence of diploid

larvae in the experiments with eggs of spawning 56 indicates that eggs of different females differ in their reaction to the cold treatment, as had been shown previously for Triturus. The total percentage of triploid larvae obtained is higher than in the experiments with *Triturus pyrrhogaster*, but lower than in the original experiments of Griffiths with *Triturus viridescens*, in which almost 100 per cent of the larvae from treated eggs were triploid. In more recent experiments with

TABLE I

Effects of refrigeration of axolotl eggs on chromosome number of young larvae
Temperature: + 1° C. to + 3° C.*
Beginning of treatment: immediately or within a few minutes after laying (fertilization).
Duration of treatment: 9 to 24 hours.

Spawning number	Total number of eggs treated	Number of Embryos	Number of larvae tail-clipped	Chromosome number		
				triploid (3N = 42)	diploid (2N = 28)	haploid (N = 14)
54	22	5	5	3	2	—
55	66	6	6	3	3	—
56	66	29	20	19	—	1
Total	154	40	31	25	5	1
Controls (total)			148	1	147	—

* In the experiment with eggs of spawning 56 the temperature rose to + 6.7° C. at the end of the treatment.

eggs of the last mentioned species, performed by Miss Rita Crotta, this figure varied from 33 per cent to 100 per cent with eggs of different females.

The occurrence of a single haploid larva was not unexpected since several haploid larvae appeared in the more extensive refrigeration experiments with eggs of *T. viridescens* and *T. pyrrhogaster*. The mechanism which is responsible for the occasional production of haploids is unknown at present. The larva was easily recognized as haploid before tail-clipping. It showed symptoms that are typical for haploid amphibian larvae, viz., dwarfing, edema, and a very characteristic pigment pattern (Figure 6). The animal was preserved shortly after amputation of the tailtip. It should be mentioned that edema and reduced viability were also noted in some of the triploid and diploid larvae developed from refrigerated eggs and thus may not have been an effect of haploidy in this case.

The single triploid found among the controls demonstrates that occasional triploid embryos originate spontaneously in the axolotl as well as in the other species of salamanders studied. In *Triturus viridescens*, where a large number of larvae have been examined during the past five years, the frequency of spontaneous triploidy is slightly over one per cent (22 of 1635 individuals, or 1.36 per cent).

Because of the cytological disadvantages of the axolotl tailtips mentioned before, no chromosome counts could be made in five preparations, and in many others the counts were approximate only. Most triploid metaphase plates were so crowded (see Figures 7 and 8) that not more than 35 to 37 chromosomes could be made out individually; the unanalyzable residue showed clearly that the actual number was somewhat higher than this, presumably triploid. In three triploid metaphase plates, however, exactly 42 chromosomes could be counted.

In view of the difficulties encountered in obtaining accurate chromosome counts, a widely used secondary criterion of polyploidy became more important, namely, the larger size of the triploid nuclei in interphase. Twenty epidermis nuclei, from each tailtip, from corresponding regions in the ventral fin, were drawn at a magnification of 630×, and the total area occupied by the 20 nuclei was determined with a planimeter. Since the epidermis nuclei in the fin are very flat discs, this area gives a fair measure of the nuclear volume. This is indicated by the fact that the average areas of 20 triploid, 20 diploid, and 20

PLATE I

FIGURE 1. Diploid larva from spawning 52, 13 mm. long, five days after tail-clipping; a new tailtip is regenerating. × 5.4.

FIGURE 2. Spontaneous triploid larva from same spawning, 12.5 mm. long; the melanophores on the head are slightly less numerous and more widely spaced than in the diploid. × 5.4.

FIGURE 3. The same diploid larva as in Figure 1, 7½ weeks later, 37 mm. long. × 2.4.

FIGURE 4. The same triploid larva as in Figure 2, 34.5 mm. long. The difference in the pigment pattern is more clearly visible than in Figures 1 and 2. × 2.4.

FIGURE 5. Triploid larva 56.2 developed from a refrigerated egg, shortly after hatching and amputation of tailtip. × 5.4.

FIGURE 6. Haploid larva 56.19, developed from a refrigerated egg, of same age as triploid 56.2, but before amputation of tailtip. The melanophores are small and numerous. × 5.4.

FIGURE 7. Triploid metaphase (42 chromosomes) in epidermis of tailfin, as seen in whole-mount of tailtip. The chromosomes are crowded, the nuclei large. × 627.

FIGURE 8. Diploid metaphase (28 chromosomes) from tailtip of control, in a slightly earlier stage of metaphase with less condensed chromosomes; for this reason the whole plate appears as large as the triploid plate in Figure 7. × 627.

FIGURES 9 to 11. Connective tissue nuclei in the fin of a triploid, a diploid, and a haploid tailtip. The differences in size and spacing of the nuclei are clearly marked. × 314.

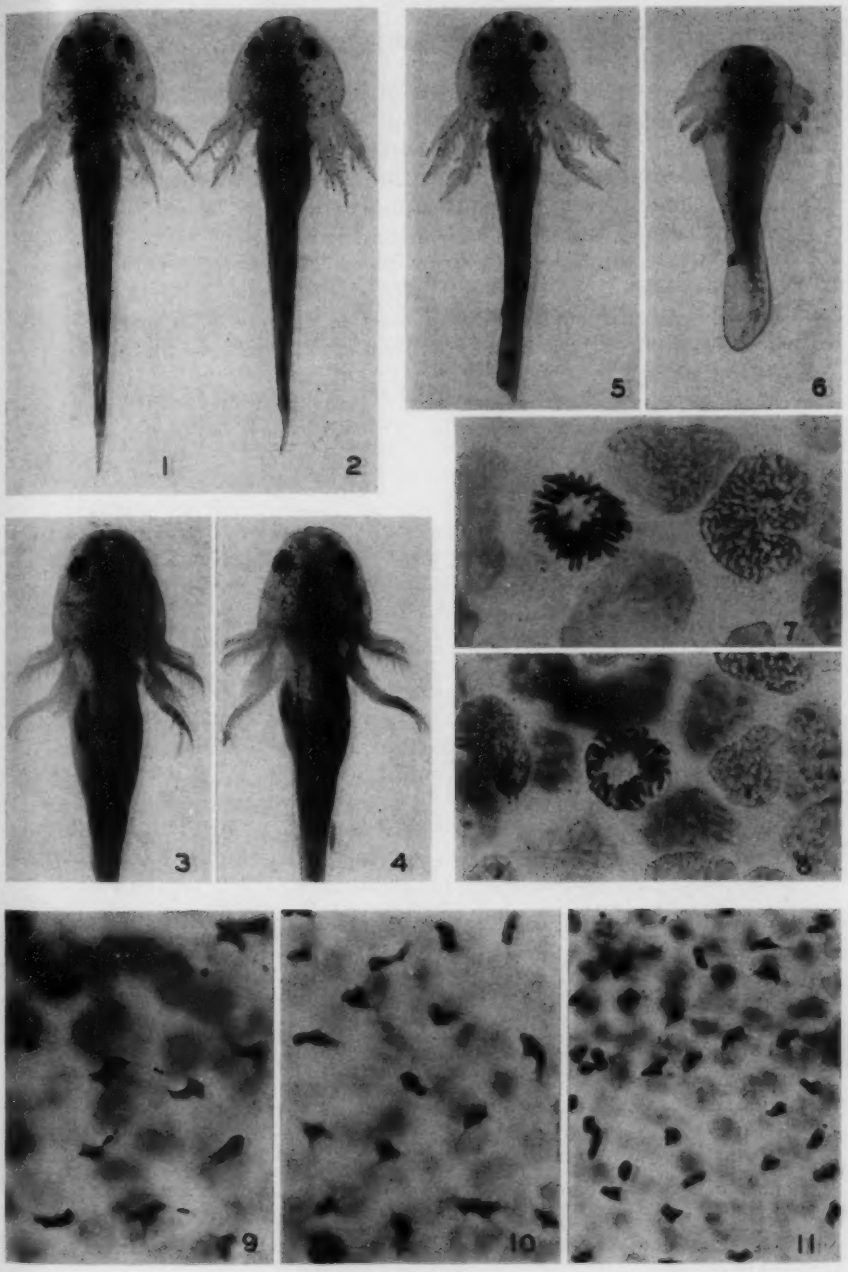

PLATE I

haploid nuclei differ almost exactly in proportion to the chromosome number (Table II). More important is the fact that although the area of 20 nuclei varies considerably between individual tailtips, the range of variation of the triploid tailtips does not overlap with that of the diploid. It is thus possible to identify a triploid tailtip from the size of the nuclei alone, without actual chromosome counts.

In many tailtip preparations red blood cells were present in capillaries in the fin. As many erythrocyte nuclei as possible were drawn at a magnification of 1433× and their greatest diameter measured. The average length of these nuclei from nineteen triploid tailtips was

TABLE II

Size of epidermis nuclei in tailfin as an index of polyploidy

	Area (in square inches) of 20 nuclei drawn at a magnification of 630×		
	Range	Average	Ratio (diploid = 2)
26 triploid tailtips	5.39 to 8.05	6.76	3.19
20 diploid tailtips	3.30 to 4.89	4.24	2.00
1 haploid tailtip	2.18	2.18	1.03

21.7 mm., the averages for the individual tailtips ranging from 20.5 to 23.5 mm. The average for four diploid tailtips was 18.2 mm., with a range of from 17.9 to 18.8 mm. The length of the erythrocyte nuclei thus offers another criterion of triploidy.

The connective tissue nuclei in the tailfin also are larger in triploid preparations and more widely spaced (Figures 9 to 11). This is easily detected when triploid and diploid preparations are viewed side by side with a comparison eyepiece. Because of their small size and irregular shape these nuclei are not favorable for actual measurements.

The classification of the larvae according to chromosome number would be greatly simplified if it were possible to recognize triploid individuals by their appearance under the dissecting microscope. In *Triturus viridescens* and *pyrrhogaster*, as well as in the lungless salamander, *Eurycea bislineata*, triploid larvae may be identified tentatively because of their usually striking pigment pattern. The individual melanophores are larger, but there are fewer pigment cells in corresponding areas (Fankhauser, Crotta and Perrot, 1942, Figure 2).

In axolotls of the white variety a number of melanophores are always present. However, they do not form a characteristic pigment pattern and can be used for a preliminary identification of triploid individuals only in some favorable cases. Following the classification

of the larvae by the tailtip method, the pigment pattern quite often served as a mark of identification (Figures 1 to 5).

Aside from the melanophore pattern, the general appearance of the nine triploid larvae which are surviving at present is normal. With one exception they are of normal size and grow at about the same rate as the controls. Seventeen of the triploid larvae developing from treated eggs were more or less abnormal and died early. However, this was true also of the five diploid larvae raised from refrigerated eggs. The abnormalities, therefore, are not necessarily connected with the change in chromosome number and must be attributed to some general harmful effect of the low temperature on the eggs, presumably on the egg cytoplasm.

Discussion

The observations reported in this paper are of general interest in several respects.

(1) They demonstrate the spontaneous occurrence of triploidy in a normal population of larvae in a fourth species of salamanders. It is noteworthy that this triploid axolotl larva developed from an egg laid spontaneously, while those previously reported in the other three species all came from eggs deposited following pituitary stimulation.

(2) The success of the three preliminary refrigeration experiments (Table I) shows that this method will be capable of producing a large number of triploid larvae, in spite of the high mortality among treated eggs.

(3) The normal body size and viability of triploid axolotl larvae confirm the observations made on the other species of salamanders. A compensation of the increase in cell size through a reduction in cell number seems to represent the typical response of the salamander embryo to an increase in chromosome number (cf. Fankhauser, 1941a).

(4) The existence of genetically different varieties of axolotls offers an opportunity to combine experimental induction of triploidy with cross-fertilization. For instance, reciprocal crosses between black and white individuals could be made and the resulting fertilized eggs refrigerated as spawned. This treatment would add a second set of maternal chromosomes and produce embryos in which either one or two of the three homologous chromosomes carry the "white" factor. It will be interesting to determine whether or not the single "black" factor in the last mentioned combination will be completely dominant over the two factors for white.

In general, the results obtained so far afford good reason to expect that it will now be possible to extend the investigations on polyploidy

beyond the period of embryonic and larval development and to combine them with breeding experiments, thus opening the way to a more detailed genetical analysis.

SUMMARY

1. One hundred and fifty-four eggs of the white race of axolotls were refrigerated shortly after fertilization, at + 1° C. to + 3° C., for from nine to 24 hours, and raised at room temperature.

2. Thirty-one larvae were obtained from refrigerated eggs. Twenty-five of these were triploid, five diploid, and one haploid. The triploid larvae presumably arose from eggs in which the exposure to cold had suppressed the second maturation division. The origin of the haploid larva is unknown.

3. Of 148 control larvae, 147 were diploid and one triploid.

4. Seventeen of the triploid larvae, as well as the five diploid larvae which developed from refrigerated eggs, were more or less abnormal, presumably because of a general harmful effect of the low temperature on the eggs. This was also responsible for the high mortality among treated eggs before and during cleavage.

5. Seven of the remaining eight experimental triploids, as well as the single spontaneous triploid, are normal in size, appearance, and viability.

6. Experimental induction of triploidy in axolotls is of particular interest because it may make it possible to raise triploid individuals to sexual maturity and to carry out breeding experiments.

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EMBRYONIC TEMPERATURE TOLERANCE AND RATE OF DEVELOPMENT IN RANA CATESBEIANA[1]

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The frogs of eastern North America, like many other organisms, have definite breeding seasons. The chronological breeding sequence is, (1) *Rana sylvatica*, (2) *Rana pipiens*, (3) *Rana palustris*, (4) *Rana clamitans*, and (5) *Rana catesbeiana*. Some overlapping in breeding time occurs, especially in seasons marked by a rapid rise in temperature, but in general the species breed in the order given. Although our knowledge of the distribution of these forms is incomplete, it appears that the same sequence holds for northern distribution. For example, *Rana sylvatica*, which is the first to breed, ranges farthest north, and *Rana catesbeiana*, which is the last to breed, has the least extensive northern distribution (Table II).

A probable basis for this dissimilarity in breeding habits and geographic distribution is to be found in certain physiological differences among the embryos that restrict each species to certain definite temperature conditions. Two of the most important effects of temperature on poikilotherms are, (1) the limiting of the range of temperatures in which vital activities occur, and (2) in modifying the rate of metabolism at different temperatures in this range. Differences that may be thought of as adaptations to both of these effects are found in frogs. *Rana sylvatica* develops normally at temperatures from 2.5° C. to 24° C., and *Rana clamitans* from 12° C. to 32° C. Thus, the early breeding, northern *Rana sylvatica* has a range of embryonic temperature tolerance that is necessitated by the low temperatures of its breeding environment. The embryos of *Rana clamitans* similarly are adapted to the warmer pond waters present when they are developing. Marked differences in rate of development distinguish these frogs. *Rana sylvatica* has the more rapid rate of embryonic growth. *Rana clamitans* is 58 per cent slower at 20° C. Thus *Rana sylvatica*, by its rapid rate of growth, offsets in part the retarding effect on its embryonic metabolism of the cold water characteristic of its breeding environment.

These questions have been discussed previously (Moore, 1939) and it was shown that *Rana sylvatica*, *Rana pipiens*, *Rana palustris*, and *Rana clamitans* fall into the same sequence when a comparison is made

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of their order of breeding, northern distributional limit, embryonic temperature tolerance, and rapidity of development. No observations on temperature tolerance, and only fragmentary data on rate of development were available at that time for *Rana catesbeiana*. In some respects this species is the most interesting as it is the last to breed and has the least extensive northern distribution. The data on *Rana catesbeiana* now to be presented are, in a limited sense, a test of the validity of the correlations previously found for the other four species. To fit the general scheme *Rana catesbeiana* should have the slowest rate of development and the highest range of embryonic temperature tolerance.

In addition a comparison will be made of the rate of development and embryonic temperature tolerance of *Rana catesbeiana* from different latitudes. Data of this nature should throw some light on evolution in the genus Rana.

Materials and Methods

The adults used in these experiments were from three localities: Rossie, St. Lawrence Co., New York; Indian Lake, Hamilton Co., in the Adirondack Mountains of New York; Schriever, Terrebone Parish, Louisiana. Some indication of the climate of these regions may be gained from data in Climate and Man (Yearbook of Agriculture, 1941). At Schriever the average July temperature is 81.9° F. (27.7° C.) and the growing season (number of days between the last killing frost in spring and the first killing frost in the autumn) is 256 days. At Ogdensburg, which is near Rossie, the average July temperature is 70.2° F. (21.2° C.) and the growing season 152 days. Indian Lake, N. Y., has an average of 64.7° F. (18.2° C.) for the month of July, and a growing season of 85 days. As *Rana catesbeiana* occurs only in the region between southern Canada and northern Mexico it will be seen that the localities mentioned above are near the northern and southern limits of geographic distribution. If adaptive changes have occurred in temperature tolerance and rate of development in different regions, material from these localities should reveal them.

Eggs were secured by means of pituitary injections. At the time of first cleavage they were placed in waterbaths, incubators, and cold-rooms. The methods used in obtaining and handling the eggs were identical with those employed in previous experiments (Moore, 1939).

Temperature Tolerance and Rate of Development

1. *Rossie, New York.*—Two females from this locality were used. In the first experiment eggs were placed at 36.2 ± 0.6°, 31.5 ± 0.9°,

TABLE I

Development of Rana catesbeiana at different temperatures. Time in hours after first cleavage. Temperatures in Centigrade.

Stage	Louisiana 14.8 ± 0.2°	Rossie 1 18.7 ± 0.4°	Rossie 2 19.1 ± 0.07°	Indian L. 19.1 ± 0.07°	Louisiana 19.8 ± 0.5°	Louisiana 24.8 ± 0.8°	Rossie 1 27.0 ± 0.1°	Rossie 2 28.2 ± 0.2°	Indian L. 28.2 ± 0.2°	Louisiana 29.9 ± 0.4°
3	0	0	0	0	0	0	0	0	0	0
8	21		12½	12½	15					
9	35	23	23½	20	18½					
10	48	30½		23		11		12½		
11	60									11½
12			35	35	35	15–21			12½	15
13	83	42½	41½–47	41½–47			18	17½	18	
14	96–121	53½	59½		48–60		21	20–23	20	19
15		68		59½					23	
16	135½–139	77½	64	64		35	30½			
17	143–192		72½–95	72½–95	83			35	35½	
18	215–288	97–127½			87–114	48	42	41	41½	35–39½
19	307–359	141–151½	144–154½	144–154½	120½	60–65	53½	47	47	
20	371	162½	157	157	131	71	56	59½	59	48½

27.0 ± 0.1°, 18.7 ± 0.4°, and 11.4 ± 0.3° (the mean and standard deviation of readings taken in the water containing the embryos). At 36.2° cleavage occurred in the animal hemisphere, but not in the vegetal hemisphere, and all embryos died before gastrulation. At 31.5°, 27.0°, and 18.7° the eggs developed normally with no indication

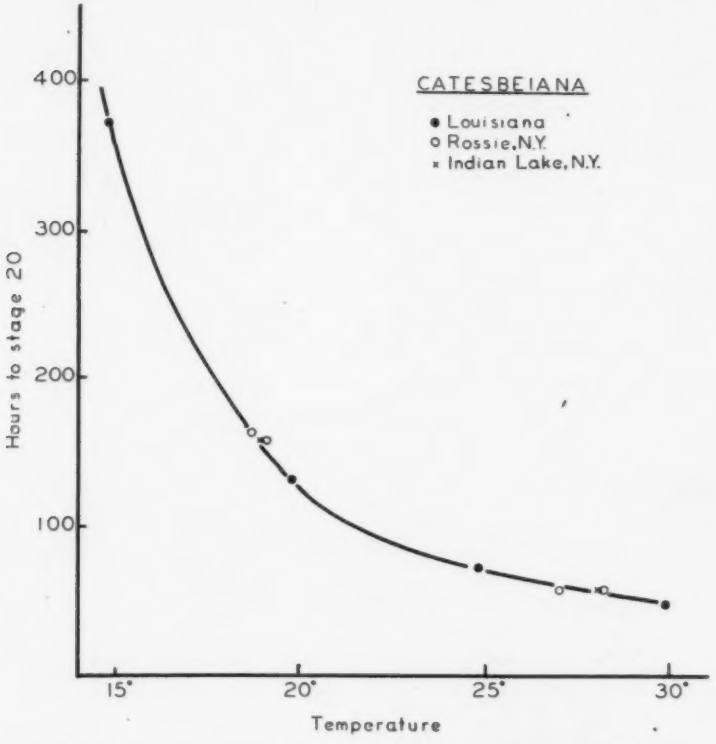

FIGURE 1. Hours required by embryos of *Rana catesbeiana* to reach stage 20 at several temperatures. Data from Table I.

of heat or cold injury. At 11.4° the eggs were definitely injured. Gastrulation was irregular and no normal embryos were secured.

In the second Rossie experiment eggs were placed at 36.4 ± 0.1°, 34.0 ± 0.0°, 28.2 ± 0.2°, 19.1 ± 0.07°, 13.9 ± 0.1°. At 36.4° and 34.0° the eggs were killed in the cleavage and blastula stages. Development at 28.2° and 19.1° was normal. At the lowest temperature, 13.9°, development was abnormal. Although some of the eggs de-

veloped to stage 20 (the stages of development are defined in Pollister and Moore, 1937), all showed injuries and abnormalities. This temperature is definitely near the normal zone and on the basis of previous experience it would be concluded that normal development would have been realized at 15°.

The rate of development was determined by examining the embryos at frequent intervals and noting the morphological stage to which they had advanced. The data in Table I represent the stage of development at the time of examination. In the case of stage 20 (beginning of gill circulation) the data represent the beginning of that stage. This stage was chosen for the accumulation of the most accurate data in order that a comparison could be made with the other species of frogs for which the stage 20 data is the best.

In the first experiment the rate of development was measured at 18.7 ± 0.4° and 27.0 ± 0.1°. At the lower temperature stage 20 was reached in 162½ hours after first cleavage, and at 27.0° in 56 hours. In the second experiment the eggs reached stage 20 in 157 hours at 19.1 ± 0.07°. At 28.2 ± 0.2° the beginning of stage 20 was missed and when examined at 59½ hours the embryos had apparently been in this stage for two or three hours. The time to reach stage 20 is indicated in Figure 1 where it will be seen that the points for both Rossie females fall on the same curve.

2. *Indian Lake, New York.*—The eggs from one female were used. This experiment was carried out at the same time and at the same temperatures as the second Rossie experiment. The temperatures employed were 36.4 ± 0.1°, 34.0 ± 0.05°, 28.2 ± 0.2°, 19.1 ± 0.07°, and 13.9 ± 0.07°. As in the case of the eggs from the second Rossie female no development beyond gastrulation occurred at 36.4° or 34.0°. At 28.2° and 19.1° the embryos were normal. At 13.9 all embryos were killed before gastrulation. It will be recalled that in the second Rossie experiment the eggs developed somewhat better at this temperature, although none were normal.

The rate of development was measured at 28.2° and 19.1°. As the second Rossie experiment was carried out concurrently a comparison of rate between these two groups of eggs could be made. This was done but no difference could be detected. At 19.1° stage 20 was reached in 157 hours after first cleavage (stage 3). The precise beginning of stage 20 was missed by several hours at 28.2°. When the eggs were examined at 59 hours they had apparently been in this stage for two or three hours.

3. *Schriever, Louisiana.*—Eggs from a single female of this locality were kept at 12.3 ± 0.1°, 14.8 ± 0.2°, 19.8 ± 0.5°, 24.8 ± 0.8°, 29.9

$\pm$ 0.4°, 33.3 $\pm$ 0.1°, and 35.6 $\pm$ 0.1°. At 12.3° the eggs were killed. At 14.8° there were many deaths and irregularities but some embryos developed fairly normally. This temperature was very close, no more than one degree below the lower limiting temperature for this species. At 19.8°, 24.8°, and 29.9° development was normal. At 33.3° and 35.6° the embryos were killed.

The rate of development was measured at four temperatures. At the lowest temperature, 14.8°, the eggs were somewhat injured but the fairly normal ones reached stage 20 in 371 hours after first cleavage. At 19.8°, a temperature well within the normal range, stage 20 was reached in 131 hours. The times necessary to reach this stage at 24.8° and 29.9° are 71 and 48½ hours respectively.

From these experiments on *Rana catesbeiana* we can set the limits of temperature tolerance. The lower limiting temperature is close to 15° judging from the development of the Louisiana eggs at 14.8° and the Rossie and Indian Lake eggs at 13.9°. The upper limit is about 32°. Development was normal at 28.2°, 29.9°, and 31.5° and abnormal at 33.3° and 34.2°.

These experiments revealed no difference in temperature tolerance in eggs from the three localities. The Indian Lake eggs (the coldest region) were certainly no more resistant at low temperatures than eggs from the other localities. As a matter of fact the Rossie eggs developed more normally than those from Indian Lake at 13.9° (the slight difference between these two groups of eggs is undoubtedly due to individual variation). Similarly eggs from Louisiana (the warmest region) were no more resistant at high temperatures than those from Indian Lake, N. Y. The data on rate of development indicate that the embryos from the three localities develop at the same speed. The data in Figure 1 form part of the same smooth curve.

Comparisons with Other Species of the Genus Rana

With the data on *Rana catesbeiana* at hand, it is possible to compare the rate of development and temperature tolerance of the five common frogs of the northeast. Observations on the other four have been given previously (Moore, 1939) but since that time more data have been accumulated.[2] These new data have been incorporated in Table II

[2] In these later experiments the temperature was much more rigorously controlled by using water baths with mercury thermoregulators instead of incubators. As a result of these new experiments it is necessary to make a slight change in the value for the upper limiting temperature of *Rana clamitans*. Previously it was found that *Rana clamitans* eggs developed normally in incubators at 33.4°. As the eggs were in water at 20° when placed in the incubator, several cleavages would have occurred before the final temperature was reached. This leads to a slight error as the tem-

where the breeding season, limit of northern distribution, temperature tolerance, and rate of development for the five species of the genus are given. As predicted *Rana catesbeiana* has the highest range of embryonic temperature tolerance and the slowest rate of growth. It will be noticed however that *Rana catesbeiana* shows no increase over the 32° upper limit of *Rana clamitans*.

We can conclude from the temperature tolerance data in Table II, that the range over which development is possible varies so that each

TABLE II

Relation between the date for the beginning of the breeding season in the New York region, northern distributional limit, embryonic temperature tolerance, and hours to reach stage 20 at 20° C. in frogs

Species	Breeding season	Northern limit	Embryonic temperature tolerance		Hours to stage 20
			Lower	Upper	
R. sylvatica	Mid March	67° 30′	2.5°	24°	72
R. pipiens	Early April	60°	6°	28°	96
R. palustris	Mid April	51–55°	7°	30°	105
R. clamitans	May	50°	12°	32°	114
R. catesbeiana	June	47°	15°	32°	134

species is adapted to definite breeding conditions. A species such as *Rana sylvatica*, which has the lowest temperature tolerance range, breeds first in the spring and maintains itself in parts of northern Canada where no other species of the genus is found. If *Rana catesbeiana* were to breed at the same time as *Rana sylvatica* its eggs would be so injured by cold that they would not reach gastrulation. On the other hand if *Rana sylvatica* were to spawn at the same time as *Rana catesbeiana* its eggs would fare no better.

It will be noticed from the data in Table II that *Rana catesbeiana* has the narrowest temperature tolerance range, the interval over which normal development is possible being 18°. For *Rana clamitans* this value is 21° and for *Rana palustris*, *Rana pipiens*, and *Rana sylvatica* 23–24°. This significance of this narrow range of temperature tolerance in *Rana catesbeiana* is not apparent. In no event is a tendency toward stenothermy characteristic of southern or summer breeding forms. However, this narrow range is correlated with small variation of water temperature in the breeding ponds of *Rana catesbeiana*. Not

perature tolerance increases rapidly during early development. When eggs are placed in water baths the final temperature is attained rapidly. *Rana clamitans* eggs do not develop at 33.8° and the upper limit is 32°, under these conditions.

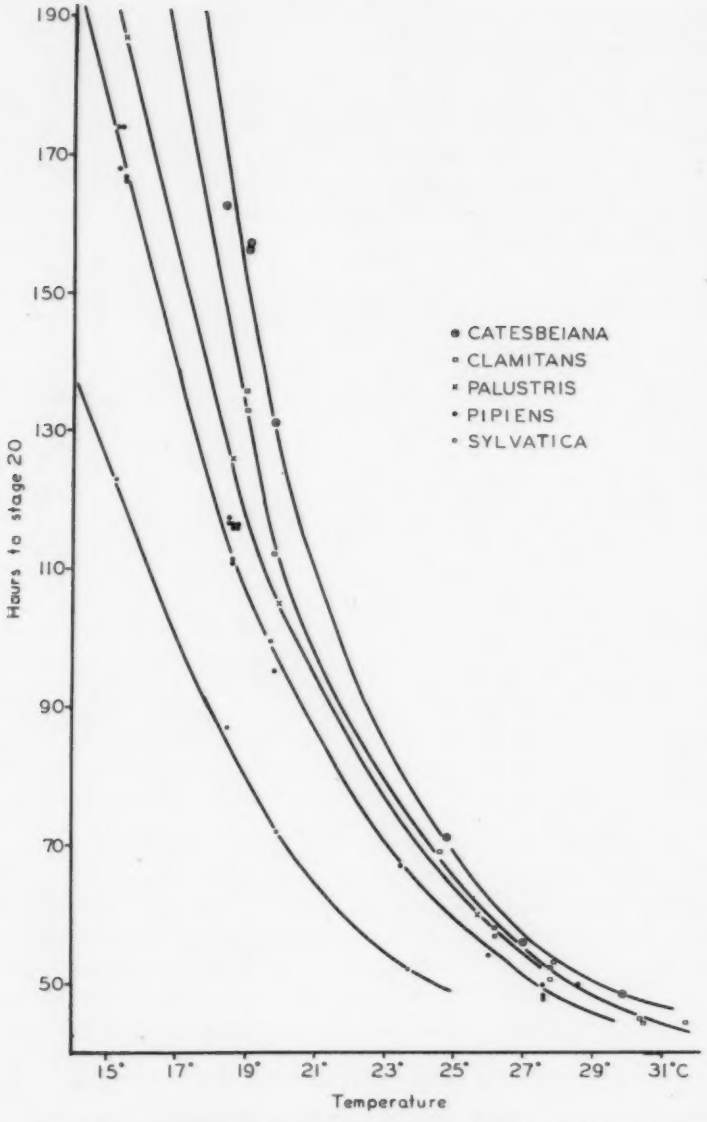

FIGURE 2. A comparison of rate of development in five species of the genus Rana. Data for *sylvatica*, *pipiens*, *palustris*, and *clamitans* from Moore (1939, and unpublished). Data for *catesbeiana* from Table I.

only does this species breed in the summer when water temperature varies less than in the spring, but in general it chooses the larger and deeper bodies of water that are more constant in their temperature than the smaller and shallower ponds, pools, and swamps where forms such as *Rana sylvatica*, *Rana pipiens*, and *Rana palustris* breed.

From Table II we can see that *Rana catesbeiana* requires the longest time to reach stage 20. That this species would have the slowest rate of development was not unexpected, as some incomplete data previously secured indicated this probable. Further, this might have been predicted on the basis of its breeding season and geographic distribution. A comparison of the rate of development over a wide range of temperatures is made in Figure 2. Little is known concerning the mechanism controlling the rate of development, but, in the final analysis, it is probably genetically determined. In certain cases it may be of considerable ecological importance. Thus we find that in general, species growing in cold regions, or in the cold months of the year, are characterized by a rapid rate of development or metabolism when compared with closely related forms in more temperate regions or breeding under warmer conditions (Fox, 1939 and earlier papers; Thorson, 1936; Moore, 1939). This rapid rate of metabolism in species characterized by low environmental temperatures enables them to offset to some extent the retarding effect of the cold surroundings on their vital activities.

Thermal Adaptation and Temperature Coefficients

Several investigators have noticed a correlation between the temperature coefficient (Q_{10} or b) and the temperature tolerance of the organism studied. Zawadowsky and Sidorov (1928) concluded from a compilation of published data on the Q_{10} for development and the upper and lower limiting temperatures for embryonic growth in several species, that the Q_{10} is greater in those forms more resistant to high temperatures. It was doubtful if much importance could be attached to this finding as the species belonged to such widely separated groups (nematodes, echinoderms, and amphibia). Therefore, these two investigators made a study on the effect of temperature on development in three ascarids and concluded that the correlation between Q_{10} and temperature tolerance suggested by their literature survey did not hold for these worms. Their choice of material for these studies was unfortunate as the difference in temperature tolerance among the three species used is so slight as to be questionable.

The following year Brown (1929) reported a correlation between the lethal temperature for cladocerans and the temperature coefficient of

their development. Those species which could withstand the highest temperatures were characterized by a Q_{10} of greater value than those species not so resistant to heat. *Moina macrocopa* with a lethal temperature of 48° had a Q_{10} of 2.39 (between 20° and 30°) and *Daphnia longispina* with a lethal temperature of 42° had a Q_{10} of 1.48. Brown gives data on seven species establishing a general relation between Q_{10} and temperature tolerance. However, the use of Q_{10} as a temperature coefficient for comparing species with different temperature tolerances is not permissible for the following reason. The Q_{10} is not constant in value but increases regularly with decreasing temperature (this is evident in the data given by Brown). Thus, if a comparison is made of the Q_{10} of the same temperature interval of two species that differ in temperature tolerance, this interval would cover a lower part of the temperature tolerance range (where the Q_{10} is normally greater) of the species more tolerant of high temperatures, and an upper part of the temperature tolerance range (where the Q_{10} is less) of the species more resistant to cold. Thus the latter species would apparently have a lower temperature coefficient even if the curves of the two were of identical shapes.

A more serious objection can be raised against Brown's conclusion that a definite relation exists between Q_{10} and temperature tolerance, namely he measured Q_{10} and temperature tolerance for two entirely different processes. His lethal temperatures were determined by placing *adult* cladocerans in water of various temperatures for one minute to test their power of survival, but his Q_{10} was for the time interval from the beginning of the first young instar to the end of the first adult instar. In most species the temperature tolerance of embryonic and adult stages is different, so unless it is shown that the cladocerans used by Brown have the same embryonic and adult temperature tolerance his conclusions are unjustified. It is therefore for two important reasons, the use of an unsatisfactory temperature coefficient, and the comparison of temperature tolerance and temperature coefficient for two different processes, that the conclusions of Brown are questionable.

In 1931 Bělehrádek placed the relationship of thermal coefficient and thermal adaptation on a somewhat more secure basis. His use of a temperature coefficient, b, which is constant over a considerable portion of the species temperature tolerance range, was a decided advance. When time-temperature data (such as those in Figure 2) are plotted on a log-log scale they form a straight line in many cases. The slope of this line is Bělehrádek's temperature coefficient b. By treating a considerable volume of data in this manner he showed the value of b was usually greater in those organisms more resistant to high tem-

perature. The processes analyzed were germination of seeds, amoeboid movement, muscular activity, heart beat, development, etc. His principal data for development come from Brown's studies on cladocerans, from which he computed b values. These species, their lethal temperatures, their Q_{10} between 20° and 30° as given by Brown, and b as computed by Bělehrádek are given in Table III. At first sight this

TABLE III

The lethal temperature, Q_{10}, and b for five species of cladocerans. Data from Brown and Bělehrádek

Species	Lethal temperature	Q_{10}	b
Moina macrocopa	48°	2.39	2.15
Pseudosida bidentata	48°	2.03	1.80
Daphnia pulex	44°	1.70	1.63
Simocephalus	43°	1.81	1.43
Daphnia longispina	42°	1.48	1.00

series appears as a striking confirmation of the postulated relationship between temperature tolerance and the temperature coefficient of development. It should be remembered, however, that a comparison is being made between the Q_{10} of rate of development of young stages and the temperature tolerance of adults. As mentioned before there is no justification for such a comparison. Further, it is difficult to understand how Bělehrádek arrives at some of his values for b. When the data for Moina are plotted on a log-log scale it is readily seen that they do not fall on a single straight line, but on two lines intersecting at 25°. The b values for these two lines are 2.3 and 1.6 (compare with values in Table III). The data for Pseudosida show such a scatter as to make them unsuitable for computing b. Bělehrádek's value of 1.80 is a rough average. The data for *Daphnia pulex* can be represented with a line of slope 1.6 to 1.8. The points for Simocephalus are even more scattered and there seems little justification for the assigned value of 1.43 since lines with slopes of 1.2 or 1.6 would be equally valid.

These data on cladocerans are the most extensive offered by Bělehrádek on a homogeneous group of animals purporting to show a relation between b and thermal adaptation in development. Even though it is not satisfactory, for the reasons given before, other data given by him covering a wide variety of processes in animals and plants seem to justify the conclusion that the value of b is greater in those species with increased resistance to high temperatures.

In view of past interest in the relation between thermal adaptation and the temperature coefficient it might be well to consider the data

for amphibia from this point of view. Observations on both rate of development and embryonic temperature tolerance are available. If the data on time to reach stage 20 (Figure 2) are plotted on a log-log scale the points in the central part of the temperature tolerance range can be represented by a straight line, but at high and low temperatures the points usually fall above this line (Moore, 1942, Figure 3). In Table IV a comparison is made of the embryonic temperature tolerance

TABLE IV

The lower limiting embryonic temperature, upper limiting embryonic temperature, and b for five species of the genus Rana

Species	Lower temperature	Upper temperature	*b*
Rana sylvatica	2.5°	24°	1.98
Rana pipiens	6°	28°	2.13
Rana palustris	7°	30°	2.30
Rana clamitans	12°	32°	2.60
Rana catesbeiana	15°	32°	2.88

and *b* for the central portion of the curve in frogs. This series is a striking confirmation of Brown and Bělehrádek's theory that the temperature coefficient increases with the adaptation of protoplasm to higher temperatures. Shorn of its specialized terminology this means that the rate of development in a species adapted to low temperature is less affected by a change in temperature than that of a species adapted to higher temperatures.

RATE OF DEVELOPMENT AND TEMPERATURE TOLERANCE IN RANA CATESBEIANA FROM DIFFERENT LATITUDES

The constant relation between rate of development and temperature tolerance on one hand and geographic distribution on the other suggests a causal connection between the two. It has long been held that temperature and moisture are the principal ecological factors limiting the distribution of amphibia. In the northeastern United States an abundance of water excludes moisture as a limiting factor. Temperature is thus left as that variable of the environment most important in controlling distribution. However, distribution is determined not by the environment alone, or by the organism alone, but by the interaction of the two. Our knowledge of the factors in the organism which are important in this connection is scanty. The difference in distribution between *Rana catesbeiana* and *Rana clamitans* obviously is not due to the presence of a dorso-lateral ridge of skin in the latter and its absence in the former. In fact the characters used in

taxonomy seem of little significance in this all-important interaction between the species and the environment which determines geographic distribution. Instead we must look for physiological differences among species that adapt each to a specific environment. In our northeast United States amphibia we must therefore search for physiological differences that are adaptations to temperature. The results so far obtained indicate that the embryonic temperature tolerance, rate of development, type of jelly mass determining the amount of oxygen available to the embryos, and perhaps egg size which is an indication of the amount of stored food material, are important in this connection (Moore, 1939; 1940; 1941; 1942).

It is necessary that physiological characters such as temperature tolerance remain fairly constant over the geographic range of the organism if they are to be controlling factors in distribution. If they were able to change easily, presumably any temperature region (with other environmental factors constant) could be colonized by any frog. Thus it may not come as a surprise that the temperature tolerance and rate of development of *Rana catesbeiana* are the same in individuals from the Adirondack Mountains and from Louisiana. These localities are near the northern and southern limits of distribution for the species and there is no evidence that in the former locality the eggs are more resistant to low temperature or in the latter locality more resistant to high temperature. With no change in temperature tolerance or other physiological adaptations we might expect a frog to be found in a fairly definite temperature region, and be excluded from colder environments in the north, and warmer environments in the south. The origin of a new population, race, or subspecies with the necessary physiological changes that would allow successful colonization of previously unfavorable regions would result in an extension of the distributional range of the species. Apparently no such change has occurred in *Rana catesbeiana* in the localities studied. These changes have occurred in *Rana pipiens* with the result that it has extended its range farther south than any of the five species found in the northeast (Moore, 1942 and unpublished).

Summary

A study of the embryonic temperature tolerance and rate of development of *Rana catesbeiana* has been made. The eggs develop normally between 15° C. and 32° C. and the time necessary to reach stage 20 at 20° C. is 134 hours. This species has the highest range of temperature tolerance and the slowest development of any of the five species of Rana common in the northeastern United States.

The significance of these results from an ecological point of view is discussed.

No difference in either rate of development or temperature tolerance of individuals from New York or Louisiana was found.

The relationship between the temperature coefficient (Q_{10} or b) of development and thermal adaptation is discussed. In frogs the value of b is greater in species adapted to high temperatures.

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SEXUAL DIMORPHISM IN THE PECTORAL FIN OF GAMBUSIA AND THE INDUCTION OF THE MALE CHARACTER IN THE FEMALE BY ANDROGENIC HORMONES[1]

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Sexual dimorphism has been found to occur among the poeciliid fishes in all of the fins except the pectoral. During a recent study of the fins of *Gambusia affinis* the writer discovered a peculiarity in structure in the pectoral fin and further study disclosed that normally it is confined to the male. Since the discovery of the character, Dr. Carl L. Hubbs has made a preliminary survey of the various Poeciliidae in the collections at the Museum of Zoology at the University of Michigan and has found that the character occurs in all the species of Gambusia, in Flexipenis and Heterophallus and to a slight extent in Belonesox. The character will be useful in taxonomic work.

The specimens used recently in a series of experiments conducted for the purpose of making a quantitative study of the effects of ethynyl testosterone and methyl testosterone upon the anal fin of the female of *Gambusia affinis* (Turner, 1942) were re-examined and it became apparent that the pectoral fin peculiarities of the male are induced in the female by the use of the androgenic hormones. The pectoral fins of the female specimens used previously were intact and since concentrations of hormone from 1 mg./2,000 cc. of water to 1 mg./20,000,000 cc. of water had been used it was possible to determine the effective limits of concentration for the development of the male type of pectoral fin in females. Some additional specimens were treated with hormone to secure a complete set of results.

It is the purpose of this paper first, to give an account of the morphogenesis of the pectoral fins of normal males and females, second, to describe the origin and character of the peculiar structure of the pectoral fin of normal males and third, to give an account of the concentrations of androgenic hormone required for the production in females of the male type of pectoral fin.

[1] This work was aided in part by a grant-in-aid from the Graduate School of Northwestern University.

The specimens used in the experiments were females 25 mm. to 27 mm. in standard length. All of the experiments were conducted at a temperature of approximately 23° C.[2]

Pectoral Fins of Juvenile Males and Females

The pectoral fins of male and of female specimens 13 mm. to 15 mm. in length are practically identical in structure (Figure 1). The fins contain 13 bony rays. Each ray is composed of an upper and a lower element which are fused together except at the base. At this stage each ray is segmented except for the basal third but an examination of a series of earlier stages reveals that the unsegmented portions are in reality parts of the rays which had been segmented previously but had undergone an extensive anchylosis of segments during their development. In indicating the number of segments in each ray the anchylosis has been taken into account and the total number of segments indicated for a ray in the tables includes the existing segments together with those formed previously and later obliterated by anchylosis. Rays 6 to 10 are bifurcated at this stage while the others are not. In later stages rays 4 and 5 become divided also while rays 1, 2, 3, 12, and 13 remain as single elements in all stages of development in both males and females. The ray-segment formula (Turner, 1941) for the fin at this stage is indicated in Table I, C, Anchylosis is most extensive in rays 3 to 7 at this stage, a little less than half of the length of each ray being solidified.

It is possible to distinguish the sexes in juvenile specimens by peculiarities of the anal fin (Turner, 1941). When the pectoral fins of large numbers of juvenile males and females, distinguished by the character of the anal fins, are compared it is found that the fifth rays of the pectoral fins of females are bifurcated while those of males are frequently undivided. However, there is a great deal of individual variation and hence this peculiarity is not reliable for distinguishing the sexes at this stage.

Morphogenesis of Pectoral Fin of Females

Development of the pectoral fin from the juvenile stage to an advanced adult stage in the female involves axial elongation and further segmentation of all of the rays, primary bifurcation of ray 4, secondary bifurcation of rays 6 to 9 and the dorsal branch of ray 10, and further anchylosis of the basal segments in all rays (Figure 2). The greatest axial elongation and addition of segments occur in rays 2 to 12 in which seven to eleven segments are added. The progress

[2] The writer is indebted to Miss Peggy Uddbom for efficient technical assistance.

of anchylosis of basal segments is greater in marginal rays than in central rays. In rays 4 to 9 the basal segments become obliterated by anchylosis for less than one-half of the lengths of the rays. In marginal rays anchylosis occurs over a greater length of the basal

TABLE I

Ray segment formulae for mature females, mature males and juvenile specimens. A. Mature 35 mm. female. B. Mature 24 mm. male. C. Juvenile male or female 15 mm. in length

A

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in secondary branches						6 | 5 | 4 | 4	5 | 5 | 5 | 5	4 | 4 | 4 | 4	3 | 3 | 4 | 3	3 | 3			
Segment number in primary branches				8 | 10	12 | 14	10 | 11	11 | 11	12 | 11	12 | 11	10 | 11	8 | 7		
Segment number in bases	6	21	20	14	12	11	11	12	12	10	10	15	9

B

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in secondary branches						4 | 3 | 3 | 3	5 | 5 | 3 | 3	4 | 3 | 4 | 3	4 | 4				
Segment number in primary branches				6 | 6	11 | 9	10 | 11	10 | 11	10 | 10	7 | 10	10 | 8	5 | 5		
Segment number in bases	5	8	22	16	14	12	11	10	11	9	11	8	3

C

Ray number	1	2	3	4	5	6	7	8	9	10	11	12	13
Segment number in primary branches						6 | 6	6 | 5	6 | 6	7 | 6	4 | 3			
Segment number in bases	4	11	13	15	15	12	12	11	9	10	9	7	3

portion, over two-thirds of the entire length being involved in ray 2. The ray-segment formula of a mature 35 mm. female is shown in Table I, A. In old females, 50 mm. in length, the anterior branch of ray 5 and the posterior branch of ray 10 are divided also and all rays have added more segments.

In each new bifurcation the new segments of a branch are somewhat shorter with the result that the rays which are branched most tend to contain the larger number of segments. In actual length, however, ray 3 which is undivided and ray 4 which contains only primary branching are as long as rays 7 and 8 which have both primary and secondary branching. The ray-segment pattern does not follow the actual fin contour which is controlled by the relative axial lengths of the rays. The arrangement of rays and segments and the contour of the anterior portion of the fin in an old female are represented in Figure 2.

Morphogenesis of Pectoral Fin of Male

The same process of axial elongation, segmentation, and bifurcation of rays and of anchylosis of the basal segments of the rays occurs during the development of the pectoral fin in the male. However, elongation and segmentation are not so extensive in the first and the last two rays. The ray-segment formula in a large adult male, 24 mm. in length, is represented in Table I, B.

A considerable degree of variation in the above formula is caused by the variation in the time of the onset of sexual maturity. Maturity may occur at a relatively early stage and, since at maturity, growth of the body and also elongation and segmentation of the rays are terminated, the small mature males have a smaller number of segments in the rays. Males may become mature when 16 mm. in length or maturity may be postponed until they are 28 mm. in length and the number of segments in the various rays will vary according to the length of the body. For example, ray 5 in a large 25 mm. mature male has thirteen segments in the primary branches while a small 19 mm. male has eight or nine segments in these branches.

During the period of development in which rays 3, 4, and 5 of the anal fin (Turner, 1941) are undergoing rapid elongation a similar process is occurring to a less extent in rays 2, 3, 4, and 5 in the pectoral fin. However, development of the anal fin is a little in advance of that of the pectoral fin. The effect of the rapid growth of the 3, 4, 5-ray complex in the anal fin is to subordinate growth in the other rays

Plate I

1. Pectoral fin of juvenile male or female, 15 mm. in length.
2. Portion of pectoral fin of an old female, 35 mm. in length. Distal two-thirds of rays 2 to 6 and the tip of ray 1 are shown.
3. Distal four-fifths of rays 1 to 6 in a mature 22 mm. male.

Rays are indicated by numbers in all figures. All drawings show the left pectoral fin from the posterior aspect. With the exception of Figure 4, rays have been separated for convenience in illustrating.

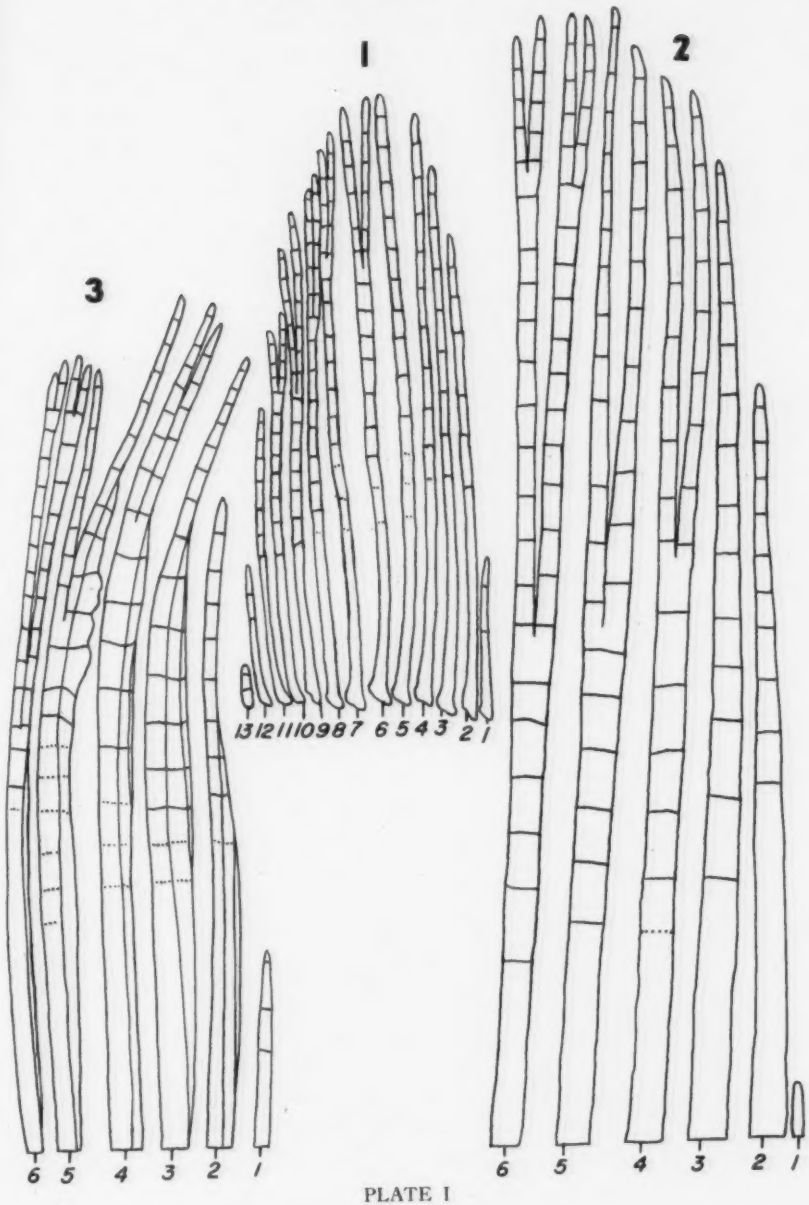

PLATE I

as well as to increase the length of the rays in the growing complex. This subordination of the growth of the rays not included in the rapidly growing complex is evident to some extent in the pectoral fin (compare rays 3 and 4 with 5 and 6 in Figures 2 and 3). Presumably the growth in rays 2, 3, 4, and 5 is a result of a susceptibility of the tissues of these rays to stimulation by a low concentration of hormone released from the developing testis. If a fish matures slowly as is usual in the large late maturing males, and a low hormone concentration is maintained over a relatively long period of time, growth in the susceptible rays is of longer duration and the differences between the lengths of the susceptible rays and the other rays is greater. In the small rapidly maturing males in which the maintenance of a low hormone concentration is of short duration and in which axial growth is quickly terminated by differentiation, the lengths of the susceptible rays are hardly greater than that of ray 6.

A second phase of dimorphism becomes apparent in the 19-segment stage.[3] In the fins of juvenile specimens each ray is composed of two bony elements which are separated at the bases but are fused together for the entire length of each ray so that they are indistinguishable. They may be separated by soaking the rays in 5 per cent potassium hydroxide. For convenience in description the normal position of the fin is disregarded and the fin will be described as though it were placed in a strictly horizontal plane. In this position each ray is composed of a dorsal and a ventral component which are fused together. From the 19-segment stage to the 26-segment stage dense, opaque tissue arises along the anterior margins of rays 2, 3, 4, and 5 and to a slight extent along the anterior margins of rays 6 and 7. Except in ray 2 only the ventral components are involved. New bony tissue is laid down upon the components of the rays. As a result the ventral components (both components in ray 2) are thickened while the dorsal components are not. Except for a short distance at the bases most of the lengths of the rays are involved. Distally the addition of new bone to the ventral components stops short of the ends of the rays by several segments. In ray 5 only the anterior branch is involved. The progress of the process is slow and at the 26-segment stage the rays involved have increased in width to the extent of about one-half of the original width. The thickening of the ventral components is accompanied by some degree of displacement of the components, the ventral member shifting into an anterior position for a distance equal to one-third of the width of the ray.

[3] Nineteen segments in the third (index) ray of the anal fin (Turner, 1941).

A third phase of differentiation begins at the 33-segment stage and progresses rapidly throughout the brief terminal period of transformation. The addition of new bony substance to the ventral components of rays 2 to 7, which up to this stage has occurred along most of the lengths of the unbranched bases, now becomes sharply localized. Beginning in an area at the point of the bifurcation of ray 5 and extending progressively to rays 4, 3, and 2 the rapid addition of new bone to each ray becomes so extensive that the newly formed bony shelf overlaps the ray anterior to it. As the shelf becomes wider it turns upward so that the area has an imbricated pattern (Figure 4). The area is quite restricted in ray 5 and covers only three or four segments of the anterior branch distal and basal to the point of the bifurcation of the ray but in rays 2, 3, and 4 the area covers a wider range of segments and is more basal in position (Figure 3). In ray 2 the dorsal as well as the ventral component is thickened. In a well developed mature male the anterior margin of the newly formed mass on ray 5 is bluntly serrated (Figure 4). During late differentiation of the fin, connective tissue forms between rays 2, 3, 4, and the anterior branch of 5 and binds the rays firmly together into a unit. The entire process of differentiation results in the partial separation of the unit from the rest of the fin and also the arching of the anterior portion of the fin.

Development of the pectoral fin in the male from the juvenile stage to completion may be summarized as follows: 1) There is axial growth, segmentation, and bifurcation of rays in the female pattern from the 12-segment to the 19-segment stage. 2) A slight separation and displacement of the dorsal and ventral components of rays 3 to 7 occurs and an addition of new bony substance to the anterior margins of the ventral components occurs at a later stage. In ray 2 new bone is added to both the ventral and the dorsal components. Rays 2, 3, 4, and the anterior branch of ray 5 grow and add new segments. 3) The process of addition of new bony substance becomes accelerated and localized in a small area within the former area involving ventral component modification. Dense connective tissue forms between the rays in the newly differentiated area and the distal parts of rays 2 to 5 are bent slightly in an anterior direction.

Modification of Female Pectoral Fin by Androgenic Hormones

Modification of the female pectoral fin proceeds in the male pattern when effective androgenic hormones are applied but the extent of the modification is limited somewhat in older specimens by the fact that the fin is already fixed in the female pattern.

The introduction of methyl testosterone into an aquarium and the maintenance for 30 days of a constant concentration of 1 mg./20,000,000 cc. of water induces in medium sized females the addition of new bone in the extensive area described in the development of the male fin. The area has the same limits and characteristics as those of the normal developing fin of the male (Figure 5). Axial growth of rays 2 to 5 does not occur at this concentration but at a concentration of approximately 1 mg./7,000,000 cc. there is some elongation. The localization and acceleration of new bone formation described under phase three in normal male development occurs at a concentration of approximately 1 mg./5,000,000 cc. The degree of differentiation at this concentration (Figure 6) is somewhat less than that in the fin of a fully differentiated male and increases in concentration do not produce further differentiation. Concentrations higher than 1 mg./5,000,000 cc. produce a condition at the ends of rays 2 to 5 in which segmentation is more rapid than axial elongation with the result that the terminal segments formed under these conditions are short.

Old females, 50 mm. in length, when treated with methyl testosterone at the most effective concentration produce modifications of the pectoral fin which are in general like those produced in medium sized females. However, there are some structural differences. In the old females ray 5 possesses secondary bifurcations in both of the primary branches before the hormone is administered. The first phase of modification in the direction of the male structure, namely, an elongation of rays 2 to 5 and the subordination of growth in the other rays is very slight. A number of thin segments are added to rays 3, 4, and 5 but due to the fact that the other rays have developed in the female pattern before hormone treatment is started they are not subordinated in growth. The second phase of modification, widening of the ventral components of rays 3 to 6 and of both components in ray 2 occurs in old females and is almost as extensive as in medium sized females. The localized widening of the ventral components of rays 3 to 5 occurs but it is not extensive and the position of the area is shifted axially.

PLATE II

4. Enlarged drawing of the imbricated area including parts of rays 3, 4, and 5 in a mature male.

5. Rays 2 to 8 of a medium sized female which has been treated with methyl testosterone at a concentration of 1 mg./20,000,000 cc. of water. Basal parts of rays are not shown.

6. Rays 2 to 5 of a medium sized female treated with methyl testosterone at a concentration of 1 mg./5,000,000 cc. of water. Basal portions of segments are not shown.

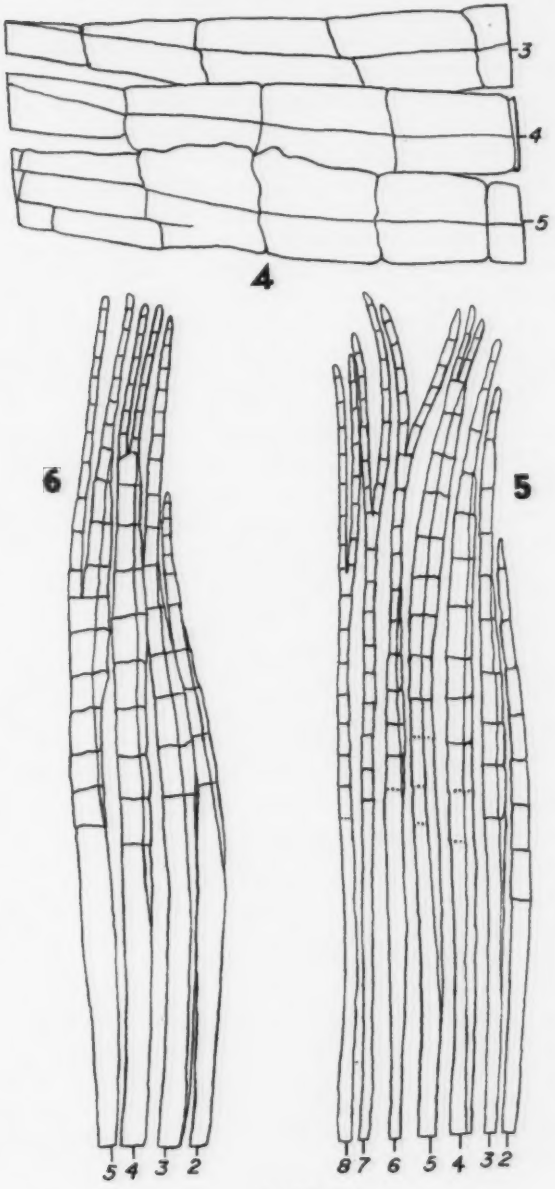

PLATE II

In normal mature males and in medium sized treated females the localized area includes that portion of ray 5 lying between a point about three segments basal to the primary bifurcation of the ray and four segments distal to the bifurcation. In old females the area includes that portion of ray 5 between a point three segments basal to the point of the primary bifurcation and 13 segments distal to the primary bifurcation. In normal mature males there is no secondary branching of ray 5 but in old females the secondary branching occurs and upon the anterior member of the anterior secondary branch the ventral component is widened and displaced for several segments. In comparing the position of the localized area in normal mature males and in old females treated with hormone it would appear that the area is oriented with reference to the point of primary bifurcation of ray 5 but that the position of the area is governed to some extent by the total length of the ray. Since in the mature male axial growth of the rays is terminalized when sexual maturity is attained, ray 5 does not increase in length after maturity. The influence of the length of the ray upon the position of the localized area is constant, therefore, and the area occurs in approximately the same position in all normal mature males. In old females, however, the ray has attained a much greater length before hormone treatment and the influence of the greater length is reflected in the shifting of the position of the localized area to a more distal position. The formation of a smaller localized area on the secondary branch can never occur in normal males because the branch is never formed. The formation of the area in treated females indicates a potentiality in males which is never realized because terminalization of growth prevents the development of the structure (secondary branch) which would become modified.

Comparison of Responses of Pectoral and Anal Fins

In the development of normal males the beginning of metamorphosis of the anal fin into a gonopodium is evident before there is any modification of the pectoral fin, but as metamorphosis of the anal fin proceeds the initial stage of the development of the pectoral fin occurs. The addition of new bony substance in the restricted area in rays 2, 3, 4, and 5 of the pectoral fin continues for some time after the gonopodial development is complete and the male is mature and reproductively functional. Apparently the structures of the anal fin which develop are susceptible to a slightly lower concentration of male hormone than any in the pectoral. The concentration required for the terminalization of development of the gonopodium is not

sufficient for the most extensive development of the pectoral fin and it is assumed that the augmented concentration required for the completion of development in the pectoral fin in normal males is furnished by the testis which continues its development after the gonopodium is completely developed.

It is not possible to produce in females, treated with different concentrations of hormone, development of the pectoral fin as extensive as that in old normal males. A concentration of 1 mg./5,000,000 cc. (methyl testosterone in water) produces maximal development of the pectoral fin in a female treated at a constant hormone level and concentrations high enough to produce early terminalization of growth and abnormalities in the differentiated structures of the anal fin (Turner, 1942) do not produce further development in the pectoral. The failure of further development in the pectoral fin of treated females may be due to the fact that the females were treated with a constant level of hormone concentration while in normal males the target organ is furnished with a continually rising concentration of hormone by the developing testis.

When ethynyl testosterone is employed, concentrations a little more than twice as great are required in order to produce the same effects as those secured with methyl testosterone but structural modifications secured by the use of ethynyl testosterone are identical with those produced by methyl testosterone.

The fact that females can be induced to develop the male characteristics in the pectoral fin indicates clearly that the female possesses the genetic factors for the development of the characters but, in the absence of an androgenic hormone, the development remains latent.

Sexual Dimorphism in Pectoral Fin of Cobitid Fishes

Vladykov (1935) has described sexually dimorphic structures in the pectoral fins in Cobitis, Misgurnus, Leptobotia, and Barbatula. In the males of different genera, from one to seven of the inner rays are enlarged. A circular bony plate occurs upon one or more rays at the bases of the fins in some species and in other species there is a conspicuous elongation of one or two of the inner rays. In all, the pectoral fin of the female lacks these features.

The differential elongation of some of the rays of the pectoral fin in the male, the thickening of specific rays and the local differentiations within the fin suggest that the situation is parallel to that in Gambusia. If females possess genetic determiners for the peculiarities but fail to develop them because of the lack of stimulation by an androgenic

hormone the characters could be brought out, presumably, by the application of androgenic hormones.

Summary

1. The pectoral fins of juvenile males and females in Gambusia are practically identical in structure.

2. In Gambusia and in a number of closely related genera a modification of the pectoral fin occurs in the male with the onset of sexual maturity. The structural peculiarities of the character consist of elongation and segmentation of rays 2, 3, 4, and of the anterior branch of ray 5, and the partial separation of the dorsal and the ventral components of these rays, a general widening of the ventral component and a development of an imbricated area by further widening of the ventral components within a localized area.

3. Females possess one or more genetic factors for the same structural characters and they can be induced to develop the characters by the application of methyl testosterone or ethynyl testosterone.

4. The first stages in the development of the characters are induced in medium sized females by treatment with methyl testosterone at a concentration of 1 mg./20,000,000 cc. of water. A concentration of 1 mg./5,000,000 cc. produces maximal development of the characters. Ethynyl testosterone produces the same results at concentrations a little more than twice as great.

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STUDIES ON THE LIFE HISTORY AND HOST–PARASITE RELATIONSHIP OF PELTOGASTER PAGURI

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INTRODUCTION

Peltogaster paguri, a rhizocephalan described one hundred years ago by Rathke from the coast of Norway, was the first species of the order to be reported from United States waters. A specimen was collected as early as 1866 by A. S. Packard, Jr., from Eastport, Maine. Whereas in European waters this parasite became the subject of a number of studies, in American waters it gained no further notice until the writer reported its rediscovery on the Maine coast and subsequently gave an account of its endoparasitic development and the rôle played by the complemental males in reproduction (Reinhard, 1939; 1942, 1942a). Concurrently, brief field notes on Peltogaster collected in the same locality were published by Walker and Pearse (1939).

In the present pages are gathered together biological and statistical notes, many being new facts about Peltogaster, others serving rather as a means of comparing the American race of this parasite with the European. The biology of *Peltogaster paguri* has been studied notably on the Channel coast of France since 1927 by Charles Pérez where the host is *Pagurus bernhardus*. On this side of the Atlantic, in an environment considerably more boreal, the parasite occurs on *Pagurus pubescens*. Such differences in host and geographical conditions should be reflected in modifications of the life history and host-parasite relationships. Moreover, to make this study as comparative as possible, comparable data for other Rhizocephala will be included wherever pertinent.

The field work on which this report is based was carried out at the University of Maine Marine Laboratory during the months of July and August, 1938 to 1941. During 1938 all the crabs were collected from nearby Googin's Ledge, where the parasite was first found by the writer on July 26 of that year. In succeeding summers attention was paid to other localities in Frenchman's Bay, and, contrary to the findings of Walker and Pearse, parasitized crabs were obtained from the

shore of Mount Desert Island and from the Lamoine shore, as well as from Racoon Cove, Skillings River, and Bar Island. Googin's Ledge, however, was favored for collecting purposes, since the percentage of infestation was higher there than elsewhere.

The host in all cases was the hermit crab *Pagurus pubescens* Kröyer, and no Rhizocephala were ever found on the other common hermit crab of this locality, *Pagurus acadianus* Benedict, although more than 300 specimens of this species were also examined.

The Percentage of Infestation

In Table I is given a summary of crabs examined with percentage of parasitism found in relation to locality and year. The entire collection of 3,092 crabs yielded 424 parasitized specimens or 13.7 per cent,

Table I

Percentage of parasitism of Pagurus pubescens with Peltogaster paguri: 3,092 crabs examined

Locality	Year	Number of crabs examined			Per cent parasitized		
		♂	♀	Total	♂	♀	Total
Googin's Ledge	1938	128	213	341	28.9	23.0	25.2
" "	1939	366	500	866	9.0	11.4	10.3
" "	1940	318	274	592	15.7	14.2	15.0
" "	1941	315	330	645	13.6	18.2	15.9
Other localities	1939	185	106	291	6.5	8.4	7.5
" "	1940	51	36	87	9.8	11.1	10.3
" "	1941	205	65	270	7.3	15.3	9.3

but of the 2,444 Googin's Ledge crabs 15.0 per cent were parasitized as compared with an infection rate of 8.6 per cent for the 648 crabs from other localities in Frenchman's Bay.

Although male and female crabs were about equally represented in the collections (1,568 males to 1,524 females) the latter were more frequently parasitized, the rate being 14.2 per cent in the case of males, 17.6 per cent in the case of females.

There is apparently in Rhizocephala a tendency toward more frequent parasitism of female than of male hosts. Extreme instances of this are reported by Pérez (1927, 1931) who found *Peltogaster paguri* parasitizing only females of *Pagurus* (*Eupagurus*) *bernhardus* at two different localities on the coast of France. More commonly the reported preponderance of infested females over infested males is on the order of the percentages given by Brinkman (1936) for *Munida sarsi*

parasitized by *Triangulus munidae:* 5.9 per cent of males and 8.3 per cent of females parasitized in one locality; 2.2 per cent of males as against 10.1 per cent of females in another locality. There are instances, however, of male hosts outnumbering female. At Roscoff, Pérez (1931a) found male *Pagurus* (*Eupagurus*) *cuanensis* more frequently parasitized by Gemmosaccus (formerly called Chlorogaster) than were the females, but the observed cases seem too few to be decisive.

The data presented in Table I show that at Googin's Ledge the percentage of parasitized females exceeded that of males in 1939 and 1941, while in 1938 and 1940 the reverse was true, but the data for other localities show female host preference for each of the three years. Taking the data all together, the percentage of parasitized females is approximately 12 per cent higher than that of males. Brinkman (1936) has suggested that the female host is a richer source of nourishment than the male and is especially more suitable for nourishment of the endoparasitic phase of the rhizocephalan, hence the preponderance of parasitized females. This explanation has much to recommend it, but the exceptions to female host preference cited above show that this principle does not always operate.

Number of Parasites per Host

As shown in Table II the total number of infested crabs amounted to 424, but 29 of these had more than one parasite. The term "scar" in this table refers to crabs that have lost the external portion of the parasite though they still contain the living roots of the parasite.

Table II

Summary of collections of Peltogaster paguri from Frenchman's Bay

Number per host	Records	Per cent
One external sac	377	
One scar	18	
Total single infestations		93.1
Two external sacs	22	
Scar and external sac	4	
Two scars	1	
Total double infestations		6.4
Three external sacs	2	
Total triple infestations		0.5
	424	100.0

The incidence of double and triple infestations is much less than would be expected on the basis of chance, i.e., either concurrent or successive infestations by more than one cypris larva. This seems to indicate some resistance on the part of the parasitized host which tends to lessen the possibility of a second infestation. Probability alone would allow 58 double infestations and eight triple infestations in the population examined, whereas the actual figures are 27 and two respectively.

Conceivably, biotic resistance would operate less strongly to prevent duplicate simultaneous infestations than superimposed infestations separated by a considerable time interval. If two infestations are acquired at approximately the same time the resulting parasites should be of about the same size; an infestation acquired some months after the first would result in two parasites of very unequal size. It is significant that 14 cases of double parasitism belonged to the class in which both external parasites were of approximately the same size, while only eight cases belonged to the other class. In Triangulus and Lernaeodiscus, according to Brinkman (1936), double infestations with parasites of equal age, as measured by size, are likewise more common than those in which the parasites differ in size.

Position of Parasite on Host

As Delage (1886) and others have noted, the external sac of Peltogaster is attached to the left side of the host's abdomen, occupying the protected space which the unparasitized female crab utilizes for carrying her eggs. A position on the right side of the abdomen would be hazardous for the parasite since this side is pressed tightly against the columella of the shell in which the crab lives. On *Pagurus pubescens* I have found the parasite regularly attached to the third abdominal segment, that is, the one that bears the first pleopod in the case of the male (Figure 1), the second pleopod in the case of the female. It is distinctive of the genus Peltogaster that the long axis of the sac is

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Plate I

Photographs of *Pagurus pubescens* Kröyer parasitized by *Peltogaster paguri* Rathke. Natural size.

Figure 1. A large male crab of 18-mm. carapace length bearing a still immature 8-mm. Peltogaster.

Figure 2. A 14-mm. male with a mature Peltogaster of average size bent in the characteristic U-position.

Figure 3. Two parasites on a 12-mm. female. Note that the smaller Peltogaster is exceptional in facing backwards.

Figure 4. Two parasites of approximately equal size (about 12 mm.) attached to a 14-mm. female crab.

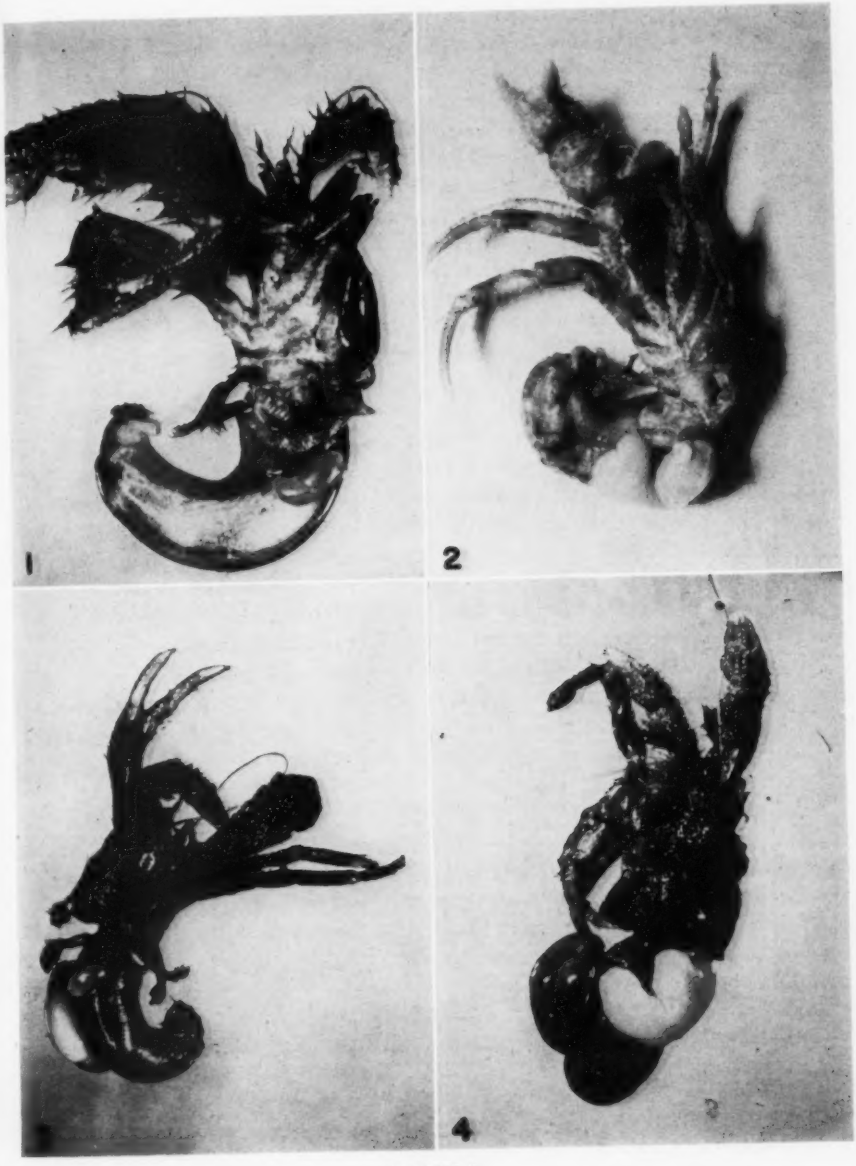

PLATE I

parallel to the long axis of the host and the mantle aperture is pointed forwards. Exceptions to the above generalizations occur primarily in multiple infestations.

Only one Peltogaster out of the 395 cases of single infestation occupied a position on the host that could be termed unusual. This Peltogaster, 10 mm. in length, was attached alongside the third instead of the second pleopod of a female crab. In no case was a Peltogaster found on the right side of the host's abdomen despite the fact that Walker and Pearse depict the animal in such a position without, however, suggesting either in text or legend that the figured specimen is remarkable. We must conclude therefore that the illustration belongs to the category of "typographical errors."

When two Peltogasters occur on the same host one occupies the normal position while the other is usually located dorsally on the same segment (Figure 4). There were only two cases in which both Peltogasters could be said to occur in approximately normal position. The dorsally located parasite, in one instance, had its long axis perpendicular to that of the host, with the mantle aperture to the left, and there were three cases where the dorsal Peltogaster was rotated through 180° so that the mantle aperture faced backwards (Figure 3).

Of the triple infestations, one consisted of a 2-mm. parasite in normal position with two others of approximately the same size arranged laterally in a line behind the first, but all three had the long axis diagonal to that of the host. The other case consisted of Peltogasters of different sizes: in normal position one of 14 mm., alongside but more dorsally one of 11 mm., and on the dorsum itself one of 3.5 mm. whose long axis was rotated 90° to the left.

Growth, Size, Color, and Sexual Maturity

It has been shown by Day (1935) and Foxon (1940) that the externae of *Sacculina carcini* reach breeding condition some six weeks or two months after their appearance. To determine the growth rate of Peltogaster, crabs with young externae were isolated in finger bowls and the size of the sac measured from time to time. A few of the experiments are here cited:

1. A male crab of 10-mm. carapace length which had a round, white, barely elevated patch on the abdomen, indicating the presence of an internans, was isolated on July 19. The Peltogaster emerged as an externa on July 30. By August 7 the sac measured about 2 mm. in length, and at the close of the experiment on August 19 the sac had reached a length of 3 mm.

2. A 4-mm. Peltogaster on a female crab of 10-mm. carapace length increased 2 mm. in length between August 7 and August 19.

3. A 4-mm. Peltogaster on a female crab of 16-mm. carapace length grew to a size of 9 mm. between July 19 and August 19.

The records from these experiments indicate that in the laboratory a young Peltogaster grows on the average approximately one mm. per week so that from six to seven weeks after eruption the external sac has reached sexual maturity.

The growth rate of the parasite, as well as the size it attains before reproducing, is dependent, to a large extent, upon the size of the host. Eggs are sometimes found in the mantle cavity of 6-mm. specimens when they occur on small hosts, while, on the other hand, very large hosts of 17- to 18-mm. carapace length have occasionally been found bearing 9-mm. Peltogasters which were not yet primiparous. On the crab of average size a Peltogaster of 7 to 8 mm. is sexually mature.

The age and sexual condition of the parasite may to some extent be gauged by the color of the living external sac. The colors and their significance are as follows:

White. The color of the newly erupted sac when only one to two mm. in length.

Red. The color of the immature sac. The ovaries have not yet ripened, and the coloration is that of the liquid contents of the visceral mass.

Apricot. The animal is mature and the color is due to ripe eggs or developing embryos.

Green. The mantle cavity is empty and the ovaries are spent. A Peltogaster rendered sterile by the presence of a female *Liriopsis pygmaea* may also be this color.

Brown. An occasional spent Peltogaster has this color which probably indicates a sac that is soon to drop off.

The size of adult Peltogasters when alive is difficult to measure exactly because of the curvature of the body and the expansions and contractions associated with ordinary respiratory movements. Moreover, when the mantle cavity is filled with eggs or embryos, the animal becomes greatly distended, and the same parasite measured after release of the nauplii will be noticeably smaller. Thus, a 15-mm. Peltogaster, after releasing nauplii, shrank to 11 mm., a 12-mm. parasite decreased to 10 mm. after shedding larvae, and a 13-mm. animal was reduced to 11 mm. Old parasites that can no longer produce eggs may live on for a time as "green spent" individuals and they likewise shrink in size. The green Peltogasters rarely measure more than 7 to 8 mm., whereas, a few weeks previously, when filled with

the last brood, many may have been in excess of 12 mm. in length. Hyperparasitism by an adult female Liriopsis also causes decrease in size.

Correlation Between Size of Host and Size of Parasite

Despite the factors just enumerated that cause marked size fluctuations, it is possible to say that the average size attained by adult Peltogasters is directly proportional to the size of the host. This is shown in Table III which gives a correlation coefficient of .601.

Table III

Correlation between size of crab and size of Peltogaster > 5-mm.

Peltogaster length in mm.	22	65	75	34	15	6	1	218
21.5				1				1
19.5			1		3	2		6
17.5					1	1		2
15.5			3	9	6	1		19
13.5			5	4	3		1	13
11.5		13	24	9				46
9.5	1	20	27	7	1	1		57
7.5	17	20	13	1	1	1		53
5.5	4	12	2	3				21
	7.5	9.5	11.5	13.5	15.5	17.5	19.5	

Crabs—carapace length in mm.

The very young Peltogasters are not included in this table because the primary concern here is with parasites of or near breeding age. It seems logical to conclude that this size relationship is based upon available food supply in the sense that the larger the host is, the larger can be the parasite which it is capable of supporting. This interpretation is in harmony with many records that could be gleaned from the literature (e.g., Boschma, 1928, p. 38) which show that on a host species of small size a particular species of rhizocephalan is apt to be dwarfed in comparison with the same species of parasite occurring on another and larger species of host. An alternative interpretation, that the Peltogasters on the larger hosts are older and therefore larger,

is unlikely in view of the fact that the size of the parasite when it becomes primiparous varies with the size of the host, as already shown.

To discover whether or not the size of the parasite is affected by the sex of the host a correlation table was prepared for the Peltogasters found on male crabs and another for those found on female crabs. A summary of these data is presented in Table IV which, as in the preceding, takes into account only the parasites greater than 5 mm.

TABLE IV

Relation between size of Peltogaster > 5-mm. and size of crabs with reference to sex of host

Crabs, carapace length	5.5	7.5	9.5	11.5	13.5	15.5	17.5
Average size P. on ♂ host	0	7.2	8.8	9.9	11.8	14.3	15.9
Average size P. on ♀ host	0	7.2	8.3	10.6	12.2	16.5	*

* Only one parasite was found on a female crab of this extreme size class; females of this size being of rare occurrence in the population.

It may be concluded from the above figures that the older female crabs in the size range from 11 to 16 mm. support larger parasites on the average than do males of the same sizes classes. Whether or not this is due to a difference in amount or quality of nourishment supplied by the different sexes is impossible to state with certainty at present.

AGE AT WHICH HOST BECOMES PARASITIZED

Charles Pérez, in a personal communication, suggested to the author that it would be interesting to determine the incidence of infestation in relation to the size of *Pagurus pubescens* in order to know at what approximate age these Pagurids are parasitized. Pérez informs me that on the coast of France *Pagurus bernhardus* is parasitized at a young age, whereas in the case of *Pagurus cuanensis* it is the adults that are parasitized. This latter host when adult is, however, of a size similar to that of young *P. bernhardus* at the age when *bernhardus* is parasitized.

My statistical data bearing on this question with reference to *Pagurus pubescens* is presented in Table V. Here it can be seen that fresh infestations with Peltogaster occur primarily in crabs of small and medium size, never in those of very large size. The greatest number of young infestations were found in adult crabs of 9 to 10-mm. carapace length, the size class which is most frequent in the population as a whole where it comprises about 40 per cent of all crabs. Because

of the small number of 5 to 6-mm. parasitized crabs the high percentage of young infestations in this class probably lacks real significance.

It would seem, therefore, that *Pagurus pubescens* presents an intermediate condition with respect to age at which parasitism takes place, since it is the young crabs and smaller adults that become parasitized. *P. pubescens* is actually a smaller species than *P. bern-*

TABLE V

Size at which crab becomes parasitized

Carapace length, mm.	Small		Medium		Large		Very large	
	5–6	7–8	9–10	11–12	13–14	15–16	17–18	19–20
Total parasitized crabs	4	27	93	97	40	18	6	1
Number with Peltogaster < 3 mm.	2	3	12	8	2	0	0	0
Per cent of parasitized having young infestations	50.1	11.1	12.9	8.2	5.0	0	0	0

hardus, but larger than *P. cuanensis*, so the hypothesis of Pérez that *Peltogaster paguri* attacks crabs of a definite size range irrespective of degree of maturity emerges strikingly confirmed.

REPRODUCTIVE CAPACITY

To ascertain the number of nauplii produced in a single brood, several lots were preserved for counting. Three 11-mm. Peltogasters yielded 9,800, 12,000, and 13,250 nauplii respectively, while one 12-mm. animal yielded 28,000. Data on the biotic potential of other Rhizocephala are non-existent.

Shortly after emission of the nauplii, the animal moults, usually casting off the external cuticle first, then the lining of the mantle cavity. A few hours later a fresh batch of eggs is released into the brood chamber. The interval between emission of nauplii and the passage of eggs into the mantle cavity for the production of the next brood was determined in ten cases, the average interval being 24 hours with a minimum of 12 hours and a maximum of 48 hours.

One of the important functions served by the moult is to free the vasa deferentia of the chitinous plug that bottles up the sperm. The manner in which the eggs are passed into the mantle cavity, the release of the sperm, and the adaptations connected with these processes have been well described by Smith (1906, p. 26). During ovulation the mantle aperture of Peltogaster is tightly closed, and the rhythmic muscular contractions of the animal cause the newly released eggs to be swished back and forth through the mantle cavity. The

jelly that later binds the eggs together has as yet not solidified, so that the ova are free to mix with the sperm as the contents of the cavity are churned together.

Numerous attempts were made to discover the time interval between successive broods but, although the Peltogasters kept under observation in aquaria promptly filled up with eggs after releasing nauplii, the embryos of the next brood never reached maturity and were aborted after various lengths of time. Judging from the degree of development attained before abortion took place it is possible to estimate the developmental period from fertilized egg to free-swimming nauplius to be approximately 30 to 40 days during the summer months. In *Sacculina carcini* the length of time between expulsion of one batch of nauplii and the next is stated to be greater than three weeks (Day, 1935) and perhaps six weeks (Delage, 1884).

Effect on the Host

The extensive literature dealing with the influence of Rhizocephala on the gonads and secondary sexual characters of host crabs, taken at face value, is full of conflicting results. But it is clear, largely from the work of Nilsson-Cantell (1926) and Brinkman (1936), that not only do various species of crabs react differently to the same parasite, but also the same species of host may be differently modified, depending on the species of parasite which infests it.

Pagurus pubescens, apart from the present investigation, has never been studied with reference to modifications caused by the presence of a rhizocephalan, but the effect of *Peltogaster paguri* on some other species of hermit crabs is known. Results of the published studies on *Peltogaster paguri* and closely related species, in so far as they deal with effects on the host, are presented for comparison in Table VI.

Briefly stated, the results of my own observations show that *Peltogaster paguri* causes complete degeneration of the gonads in the female *Pagurus pubescens* but does not cause castration of the male. Moreover, there is no appreciable modification of the secondary sexual characters in either sex.

To ascertain the effect on the gonads a large number of parasitized crabs were dissected in the fresh state. In addition, ten parasitized individuals of each sex were cut into serial sections and approximately the same number of normal individuals were sectioned for controls. Females bearing an external Peltogaster had ovaries so reduced that merely a trace remained which could be detected only with difficulty. Females, however, infected with the internal stage of the parasite, still had large ovaries with nearly as many eggs present as in the normal

TABLE VI

Effects of Peltogaster paguri and related species [1] on various hosts. A tabulation of cases reported in the literature

Parasite	Host	♂ Gonad	♀ Gonad	♂ Pleopods	♀ Pleopods	Authors	Remarks
Peltogaster paguri	*Pagurus bernhardus*	Atrophied	Atrophied (?)	Unmodified	Masculinized	Giard, 1887	Relatively few cases studied.
" "	" "			Unmodified	Masculinized	Guérin-Ganivet, 1911	Only six parasitized crabs examined.
" "	" "	Atrophied				Mouchet, 1931	No statement regarding external characters or effect on female.
" "	*Pagurus cuanensis*			Feminized	Unmodified	Nilsson-Cantell, 1926	Condition of gonads not noted.
" "	*Anapagurus chiroacanthus*			Unmodified	Unmodified	Nilsson-Cantell, 1926	Notes atrophy of male copulatory organ.
" "	*Pagurus pubescens*	Unaffected	Atrophied	Unmodified	Unmodified	Reinhard, this paper	
Peltogaster curvatus	*Pagurus meticulosus*	Atrophied	Atrophied	Feminized	Unmodified	Potts, 1906	
" "	*Pagurus prideauxii*	Atrophied	Atrophied	Feminized	Unmodified	Potts, 1906	
" "	" "	Atrophied				Mouchet, 1934	One specimen examined histologically.
Peltogaster sp.	*Pagurus samuelis*	Atrophied	Atrophied	Feminized	Masculinized	Shiino, 1931	Many cases, statistically treated, but species of parasite unfortunately not determined.

[1] References to "Peltogaster sulcatus" are omitted because this animal, now known as *Gemmosaccus sulcatus*, is a gregarious species, differing in a number of important respects from the genus Peltogaster.

animal. The male gonads, on the other hand, even when examined microscopically, showed no clear-cut evidence of degeneration and the spermatophores in the vas deferens appeared normal. Normal, active sperm were repeatedly extracted from the vasa deferentia of live parasitized males.

In number, shape, and ornamentation of the pleopods, as well as in position of the reproductive openings, the males and females of *Pagurus pubescens* are readily distinguished.

The male hermit crab possesses three pleopods while the female has four. No parasitized male with a supernumerary pleopod on segment 2 was found, but at least three normal males were noticed with this extra appendage. Two of these anomalous crabs were sectioned but the testes were found to be normal and there was no evidence of past or present infection with parasites.

In females, the rami of the pleopods are subequal in length, but in males the external ramus is considerably longer than the internal ramus. Since no feminizing of male pleopods or masculinization of female pleopods under the influence of Peltogaster could be detected by inspection alone, it was decided to try exact measurements in the expectation that the endopod-exopod ratio used by Shiino (1931) might show a difference between normal and parasitized animals. The pleopods of approximately 50 crabs of each sex, equally divided between normal and parasitized individuals, were examined statistically, but since no significant differences appeared to be materializing, this study was abandoned.

A further sex difference in the pleopods is seen in the bouquets of hairs that occur on the protopod. In many Pagurids it is stated that only the female pleopods have tufts of hairs on this portion of the appendage. The specimens of *Pagurus pubescens* that I examined show setae on the protopod of both sexes although these hairs are very numerous in the case of the female and sparse in the male. A comparison between normal and parasitized animals failed to show any increase in the number of hairs for parasitized males or decrease for parasitized females.

Finally, the tuft of setae that occurs on the outer margin of the endopod of a female abdominal appendage was used as a criterion. This cluster of hairs is normally absent in the case of the male. It occurred, however, on the pleopods of two unparasitized males, but on none of the parasitized specimens examined. It was never absent, so far as noted, from the pleopods of parasitized females.

The natural and not infrequent occurrence of intersexual and other types of anomalies in Crustacea makes it hazardous definitely to

ascribe such variations to the action of a parasite, when the condition is present in a particular host, unless sufficient cases are at hand to eliminate the possibility of coincidence. Among the crabs parasitized by *Peltogaster paguri* there is in my possession one female of 11-mm. carapace length that exhibits what is undoubtedly a very rare condition, the presence of a pleopod on the right side of the abdomen in addition to pleopods, normal in number and structure, on the left side. This extra appendage, well developed and female in type, occurs on the fourth segment. I see no reason, in view of the large number of parasitized females examined by me that proved to be unmodified, to ascribe this condition to the presence of Peltogaster.

Summary

Of 3,092 *Pagurus pubescens* examined, 13.7 per cent were found to be parasitized by *Peltogaster paguri*, the incidence of infestation being somewhat higher in female than in male crabs. Considerable biotic resistance on the part of the parasitized host is indicated by the small number of double and triple infestations.

The external sac, which reaches sexual maturity in from 6 to 7 weeks after eruption, produces from 10,000 to 28,000 nauplii, and successive broods may follow one another at approximately 30 to 40 day intervals. The average size attained by adult Peltogasters is directly proportional to the size of the host, female crabs supporting, on the average, larger parasites than do the males.

The age at which parasitism takes place depends on the relative size of the different host species, and is not necessarily related to the degree of host maturity.

Peltogaster causes complete degeneration of the gonads in the female *Pagurus pubescens* but does not cause castration of the male. Secondary sexual characters are not appreciably modified.

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SOME EFFECTS OF COVERING THE PERISARC UPON TUBULARIAN REGENERATION

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Recent experimental work on Tubularia has emphasized the importance of the uncovered ends of sectioned stems as loci of metabolic exchange. However, there have been some indications that the perisarc is not wholly impermeable. Thus Barth (1940) reports that as many as 50 per cent of stems shaken in Warburg manometers developed hydranth primordia even though both ends were ligatured. Although the stems in question had been subjected to environmental conditions which were hardly normal, the results suggested that the question of metabolic exchange through the perisarc should be investigated and its possible rôle in regeneration determined.

The problem was attacked by studying the effects of covering the middle of the stems for different distances, thereby reducing to a greater or lesser degree metabolic exchange through the perisarc without interfering with exchanges through the ends of the stems. As will be seen later, the findings show that, under conditions of normal (or at least nearly normal) oxygen and metabolite concentrations, varying the opportunities for metabolic exchange through the perisarc affects regeneration at the distal surface to a small degree. However, the effects of exchanges through the perisarc are definitely secondary as compared with those through the distal cut surface.

Material and Methods

The colonies from which the stems were selected were collected from the Oceanographic wharf at Woods Hole, Massachusetts, and from the University of Maine Marine Laboratory float at Lamoine, Maine.[2] The stems were selected for similarity with respect to diameter and general appearance. The distal cut in all cases was made 5 mm. behind the hydranth.

Capillary tubing with outside and inside diameters of 2.5 mm. and 1.0 mm. respectively was cut into lengths of 2.5, 5, 10, and 20 mm. for

[1] Now in the Division of Anatomy of the University of Tennessee.

[2] The work at Woods Hole was performed while enjoying laboratory facilities financed by the Faculty Research Fund of the University of Michigan.

the various experiments performed. These were attached with plasticine to one edge of strips of window glass 2 cm. wide and 20 cm. long which in turn were fastened to the bottoms of 10-inch finger bowls. Each finger bowl contained two glass strips spaced about 5 cm. apart and on each strip were 13 of the tubes (Figure 1). The finger bowls, filled with sea water to a point where the tubes were just covered, were placed on a water table with temperatures recorded several times a day.

The first ten stems to develop the constriction separating hydranth from stem were measured and the remaining three were discarded. All stems were removed from the tubes when the first primordium was measurable. This was found necessary because disturbances in the water produced in removing the first stems sometimes caused others to shift their positions in the tubes.

In this connection it should be noted that although they could not be given the benefit of circulating water, the stems nevertheless re-

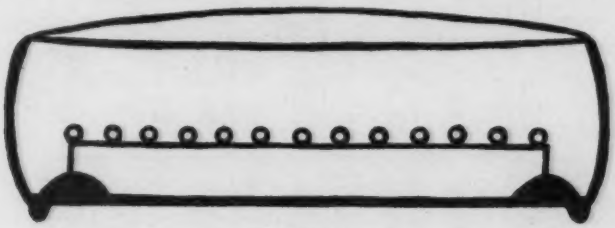


FIGURE 1

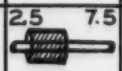
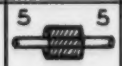
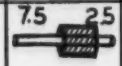
generated under favorable conditions. In a preliminary experiment a control set of stems lying on the bottom of the container required 1.5 hours longer to develop and averaged 6.8 units shorter than did an experimental lot in tubes which covered 50 per cent of the stem. It may be noted that in the case of the stems lying on the bottom the opportunities for metabolic exchange were limited along one surface by contact with the substrate, while those in the tubes, surrounded by water on all sides and located close to the surface, were regenerating under no such limitations.

The favorable conditions under which the experments were carried out were reflected by the unusually long primordia that developed. A number of the stems were found to have primordia 2.5 mm. or more long and one of 2.8 mm. length was recorded.

Experimental

Barth (1938a) reported an experiment in which a collar placed near or over the distal end of a tubularian stem was found to prevent hy-

TABLE I

		Distal Hydranths				Proximal Hydranths			
Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8	Col. 9	Col. 10
Expt. 1	Diagram	Percent	Diameter*	Time	Length*	Percent	Diameter*	Time	Length*
Lot 1		100	25.9	64.6 (55-72)	51.9 (65-35)	90	20.1	66.7 (55-77)	50.0 (75-28)
Lot 2		100	25.1	43.6 (42-45)	68.6 (81-51)	60	19.5	66.1 (64-68)	36 (46-32)
Lot 3		100	25.1	42.3 (41-44)	86.6 (102-58)	50	18.8	84.6 (67-96)	31.6 (37-22)
Lot 4		100	25.6	41.8 (41-44)	87.7 (103-78)	50	21.2	78.8 (67-97)	35.2 (51-22)

15 mm. stems with 5 mm. covered by glass tubes. Temperature range 13.4° to 14.6° C. All stems were removed from tubes after 41½ hours.

* 1 unit = 0.445 mm.

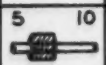
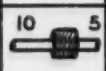
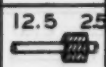
dranth regeneration at that surface. Therefore, before studying the problem mentioned above, it was necessary to determine how far from the distal end a tube, of the diameter and thickness employed, must be placed in order to eliminate its action in reducing exchanges through the end of the stem. To determine this, two experiments were performed (Table I). In the first experiment four lots were cut. In Lot 1 the distal end of each stem was arranged flush with one end of the tube. In Lot 2 the distal ends of the stems projected 2.5 mm.; in Lot 3, 5 mm.; and in Lot 4, 7.5 mm. from the ends of the tubes.

From the tabulated results it may be seen that with respect to regeneration of the distal hydranth there was no appreciable difference in mean length or mean time between Lots 3 and 4. Between Lots 2 and 3, however, there was a distinct increase in mean length and between Lots 1 and 2 there was both an increase in length and a decrease in time (columns 5 and 6). That the stems in Lot 1 regenerated at all may be attributed to their removal from the tubes after 41½ hours. On the other hand, it should be noted that regenerative processes were not completely stopped during the period that the ends were covered. Subtracting the time they were covered (41.5 hrs.) from the mean time of measurement of the primordia (64.6 hrs.) we find that after removal from the tubes primordia regenerated in only 23.1 hours. This is approximately 19 hours less than were necessary for regeneration in Lots 3 and 4. Thus it would appear that the earlier steps in the regeneration process were passed through while they were still in the tubes.

Referring to the second half of the table we see that interfering with metabolic exchanges at the distal surface allows an increased percentage of proximal hydranths to regenerate (column 7). These hydranths appear to be larger and to develop more rapidly than when the distal hydranth has not been inhibited in this manner (column 9) although, because of the greater variability characteristic of the proximal hydranth, the latter findings are not as clear cut as in the case of the distal hydranths.

In the first experiment the time of constriction was slightly less and the length of the primordia slightly more in Lot 4 than in Lot 3. The experiment was repeated with longer stems which permitted placing the collars at greater distances from the distal end. This was done in order to determine if the difference was real, and if so, whether it was a result of the proximity of the tube to the proximal end or of the distance from the distal. In Experiment 2 (Table II) 20 mm. stems were used and the collars were placed 2.5 mm., 5 mm., 7.5 mm., and 12.5 mm. from the distal end. In this experiment, performed in Maine,

TABLE II

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6	Col. 7	Col. 8	Col. 9	Col. 10	Col. 11
Expt. 2	Diagram	No. Measured	Av. Diameter ★	Av. Time (Hours)	Av. Length Primordia ★	Expt. 3	No. Measured	Av. Diameter ★	Av. Time (Hours)	Av. Length Primordia ★
Lot 1		8*	32.0	39.3 (37–42)	82.6 (67–103)					
Lot 2		10	27.3	39.3 (37–42)	90.9 (76–104)	Lot 1	10	25.4	58.0 (58)	106.0 (93–118)
Lot 3		9*	26.8	39.2 (37–42)	91.1 (71–101)	Lot 2	10	26.4	58.1 (58–59)	104.2 (98–113)
						Lot 3	10	25.5	58.2 (58–59)	105.7 (94–119)
Lot 4		10*	30.1	34.4 (37–41)	92.8 (78–104)	Lot 4	10	26.1	55.5 (55–57)	109.3 (101–123)

20 mm. stems with 5 mm. covered by glass tubes.
Experiment 2. Temperature range 13.6°–14.6° C. All stems removed from tubes after 37½ hrs.
Experiment 3. Temperature range 12.8°–14.0° C. All stems were removed from tubes after 58½ hrs.
* Because of vibrations of the building 5 stems in Lot 1, 4 in Lot 3, and 2 in Lot 4 shifted position and therefore were discarded.
★ 1 unit = 0.445 mm.

jarring of the building resulted in shifting the position of a number of the stems. In Lot 1 five, in Lot 3 four, and in Lot 4 two stems had to be discarded for this reason. The variation in average diameter of the stems which were measurable also detracts from the value of this experiment. However, the results were essentially the same as in Experiment 3 with reference to the effective inhibitory range of the collars. In both Experiments 2 and 3 there was no difference in time or length of the primordium between the 5 mm. and 7.5 mm. lots and in Experiment 3 increasing the distance to 10 mm. likewise did not increase regeneration. This shows that the increased regeneration in the 7.5 mm. lot of Experiment 1 was not the result of the greater distance from the collar

TABLE III

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Expt. 4	Diagram	No. Measured	Av. Diameter*	Av. Time (Hours)	Av. Length Primordia*
Control		10	31.6	40.4 (38-35)	84.7 (62-95)
Experimental		10	31.5	39.3 (38-41)	91.7 (83-99)

20 mm. stems with 10 mm. covered by glass tubes. Temperature—13.6°–16.5° C.
* 1 unit = 0.445 mm.

to the distal end. On the other hand, it should be noted that in Lot 4 of all three experiments the proximal ends of the pieces were 2.5 mm. from the collar, and in each case they showed increased distal regeneration over Lot 3. Since it was shown by Experiments 1 and 2 that exchanges through the distal ends were influenced by collars 2.5 mm. from the distal ends, it appeared highly probable that a similar condition would obtain with respect to the proximal end.

The question of the possible beneficial effect upon distal regeneration by interfering with exchanges through the proximal cut surface was investigated next. Isolation of the distal from the proximal surface by a ligature is known to increase distal regeneration (Barth, 1938b). However, it does not necessarily follow that inhibiting proximal regeneration without stopping circulation would produce the same effect. A priori it might be predicted that the oxygen deficiency and increased

metabolite concentration which might develop at such a covered end might be distributed by the coelenteric currents and produce inhibition at the opposite end.

In Experiment 4 20 mm. pieces were placed in tubes 10 mm. long. In the control lot the stems projected 5 mm. from each end and in the experimental lot the proximal ends of the stems were arranged flush with one end of the tube (Table III, column 2). A glance at columns 5 and 6 of Table III shows that the experimental treatment resulted in an actual increase in distal regeneration with respect to length of the primordia and time at which constriction was first visible.

In this experiment the total amount of stem covered did not influence the result since it was the same in both experimental and control lots. Likewise the result cannot be attributed to the increased distance from the distal end of the stems to the tubes, since in the previous experiment it was found to have no effect between 5 mm. and 10 mm. Therefore, it is concluded that the increased regeneration of the experimental over the control lot was the result of covering the proximal end. It also lends added support to the conclusions arrived at from a comparison of Lots 3 and 4 of the first three experiments, namely, that the increased distal regeneration observed in Lot 4 of each experiment was the effect of the nearness of the collar to the proximal surface, thereby reducing in some manner its inhibitory effect upon distal regeneration.

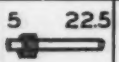
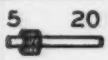
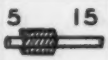
By allowing 5 mm. of stem to project from the ends of the tubes it was now possible to test the effects of covering various amounts of perisarc. In Experiment 5 a comparison was made between stems covered with 5 mm. and with 10 mm. tubes (Table IV). No difference in time of constriction was observed (column 5), but with respect to length, covering an additional 5 mm. of stem resulted in a decrease of 6 units (0.126 mm.). This represents a decrease of 8.6 per cent over the 5 mm. covered stems.

The experiment was repeated (Experiment 6) with tubes 2.5, 5, 10, and 20 mm. long. In this case the stems were longer (30 mm.) and larger, the temperature was lower (temperature range 13.8° to 16° compared with 18.0° to 18.6° C.) and the primordia which developed were larger. It is not possible to say how the differences in the two experiments correlate with the differences in the result, but it is readily observed that in Experiment 6 the tubes were far less effective in reducing the size of the regenerate.[3] In Experiment 5 doubling the area

[3] When saturated, seawater at 18° C. has about 7 per cent less oxygen, about 11 per cent less "free" carbon dioxide and about 2 per cent less "combined" carbon dioxide than it does at 14° C. (calculated from Table 235 in "Properties of Ordinary Water Substance," N. E. Dorsey, 1940, Reinhold Publishing Corp., 667 pp.). It is therefore apparent that stems regenerating at lower temperatures find it easier to

covered resulted in an 8.6 per cent decrease in average length of the primordia. In Experiment 6, on the other hand, it took a ratio of four to one to produce an equivalent reduction. Increasing the length of

TABLE IV

Col. 1	Col. 2	Col. 3	Col. 4	Col. 5	Col. 6
Expt. 5	Diagram	No. Measured	Av. Diameter*	Av. Time (Hours)	Av. Length Primordia*
5 mm.		10	24.9	28.8 (28–31)	81.7 (68–92)
10 mm.		10	25.0	28.8 (27–31)	75.7 (47–101)
Expt. 6					
2.5 mm.		10	29.9	40.2 (39–42)	102.7 (92–110)
5 mm.		10	30.0	40.5 (39–42)	99.9 (92–111)
10 mm.		10	30.1	40.5 (40–42)	94.0 (89–101)
20 mm.		10	30.6	40.7 (40–42)	93.0 (70–108)

Experiment 5. 20 mm. stems with the distal 5 mm. projecting from the ends of the tubes. Temperature range 18.0°–18.6° C.

Experiment 6. 30 mm. stems with the distal 5 mm. projecting from the ends of the tubes. Temperature range 13.5°–16.0° C.

*1 unit = 0.445 mm.

obtain oxygen and to eliminate carbon dioxide than at higher. In addition, lower temperatures reduce the respiratory requirements and the speed of synthesis of new tissues. All these factors would tend to decrease the effectiveness of tubes at lower temperatures, but at present it is not possible to say which are of major and which of minor significance.

the collar from 2.5 to 10 mm. produced an 8.5 per cent reduction in length and from 5 to 20 mm. gave a 7.9 per cent decrease. Doubling the surface covered gave only the following percentage reductions: from 2.5 to 5 mm.—2.7 per cent; from 5 to 10 mm.—5.9 per cent; from 10 to 20 mm.—1.1 per cent. An eight-fold increase in area covered was sufficient to decrease the length by only 9.4 per cent (from 102.7 to 93.0 units). It is of interest that in the 20 mm. lot, two-thirds of the entire surface area of the perisarc was covered. When such is possible with so little effect upon time and primordium length it is obvious that under the conditions of the experiment the metabolic exchange through the perisarc is definitely subordinate to that through the ends of the stem.

Discussion

These experiments were designed to determine whether and to what extent metabolic exchange through the perisarc affects the time of appearance and the length of the regenerating hydranth primordium. Because of the necessity of exposing at least 5 mm. of stem at each end of the tubes, it was not possible to test short, i.e. very young, stems. However, with this limitation, it was noted that even when large areas of the stem were covered there were relatively slight inhibitory effects. In one experiment there was a decrease of only 9.4 per cent in length when the glass tubes covered two-thirds as compared with one-twelfth of the area of the stem, an eight-fold difference (calculated from Experiment 6). In another (Experiment 5) run at a higher temperature and with shorter stems, doubling of the area covered gave a 10.2 per cent decrease in primordia length.

On the other hand, the ends of the stems showed marked susceptibility to the presence of the tubes. A regenerative decrease of similar order of magnitude as above was produced by arranging the stems with 2.5 mm. of the distal ends extending beyond the tubes. In this case the reduction in length was 20.8 per cent and 9.1 per cent (calculated from Experiment 1, Lots 2 and 3, and Experiment 2, Lots 1 and 2 respectively). Complete inhibition when stems are left with their ends flush with the tubes has already been reported (Barth, 1938a; Rose and Rose, 1941) and was confirmed by my own unreported preliminary experiments. In the present experiments the 40.0 per cent decrease in length of the hydranths of Lot 1 over Lot 3 in Experiment 1 represents an incomplete recovery following removal from the tubes.

The effects on time of regeneration were generally so slight that they do not warrant any discussion excepting the comment that it appears questionable whether data on tubularian regeneration should be recorded as "regeneration units (R. U.)" calculated from a formula in

which length is divided by time (Barth, 1938b) and based on the implied assumption that length and time are inversely related to each other. The present experiments cast doubt on the validity of this assumption, since in some cases length varied while time did not. A case in which such a relationship cannot hold was recently reported in abstract by Moog (1941) who found that stems at lower temperatures required longer to regenerate but produced larger primordia than did those at higher temperatures.

It should be pointed out that the tubes interfered with diffusion out of as well as into the stems. Barth who used somewhat similar tubes (1938a) failed to recognize this possibility and attributed the inhibition produced entirely to oxygen deficiency. Similarly in the experiments of Zwilling (1939), in which regeneration followed the localized removal of perisarc, it appears highly probable that the operation allowed the escape of inhibitors as well as the entrance of oxygen.

The necessity of oxygen for regeneration has been clearly demonstrated (Torrey, 1912; Barth, 1937, 1938a, 1940; Miller, 1937, 1939; Rose and Rose, 1941). However there is also growing evidence of inhibitory substances which must be allowed to escape from the stem in order for regeneration to proceed at a maximal rate. A thermally unstable inhibitor has been found by the Roses. Miller (1939) found carbon dioxide to be a powerful inhibitor, which even when mixed with oxygen in proportions of 10 per cent to 90 per cent would completely prevent regeneration. The fact that carbon dioxide combines with water to produce carbonic acid would suggest that the inhibitory action of the gas might be a result of the increased acidity of the solution. The possibility of inhibition by acidity was first suggested by experiments of Loeb (1904) concerning which he made the following statement: "One gains the impression that in the tubularian stem an acid is formed which delays growth if it is not neutralized" (p. 147). This suggestion seems to have passed unnoticed by later experimenters. Child (1931) found evidence of inhibitory action by lowered pH and Komori (1933) reported an experiment in which distal ends exposed to pH 6.0 failed to form hydranths while ten of the thirteen proximal ends of the same pieces exposed to pH 8.45 did. Work this past summer (Miller, 1942b) has shown that there actually is an increase in acidity of the coelenteric contents of stems enclosed in tubes used in the experiments reported in this communication.[4] Whether or not this acidification may be attributed entirely or largely to carbon dioxide accumu-

[4] The accumulation of pH lowering substances in tubes in which stems have remained for 96 hours has just been reported, as well as further evidence on the inhibitory action of low pH. (Goldin, A., 1942. *Biol. Bull.*, **82**: 243–254.)

lation has not been determined, but it appears likely that it plays a major rôle.

Already reported experiments have indicated that the oxygen content of the middle of the coelenteron is not affected by increasing that of the external environment even though regeneration was stimulated by the treatment (Miller, 1940). When these findings are considered in the light of the fact that the oxygen consumption of the tubularian stem varies with the oxygen tension (Barth, 1940), it becomes evident that any oxygen that penetrates the perisarc is utilized by the subadjacent ectoderm before reaching the entodermal ciliary mechanism or the coelenteric contents. Likewise, it follows that any localized diminution of oxygen in the milieu, such as would occur within the tubes, would be matched by a corresponding decrease in oxygen uptake by these same cells, again without affecting the coelenteric contents. It is therefore suggested that the inhibitory action of the tubes, when placed with at least 5 mm. of stem projecting from the ends, was not the result of anoxemia.

On the other hand, an increase of acid producing metabolites has been noted in stems placed in the tubes (Miller, 1942b) and acidity is known to inhibit tubularian regeneration. It is therefore tentatively suggested that the degree of reduction in length of primordia of stems regenerating in the longer tubes represents the extent to which the elimination of inhibitors is accomplished by the unbroken perisarc.

Summary

1. Pieces of Tubularia stems were placed in glass tubes with outside and inside diameters of 2.5 mm. and 1 mm. respectively in order to determine the rôle in regeneration played by metabolic exchange through the perisarc.

2. Using tubes of the same length it was found that when the distal ends of the stems projected less than 5 mm. from the tubes regeneration was decreased at the distal surface. When the proximal ends projected less than 5 mm. from the tubes there was an increase in distal regeneration.

3. Using tubes of different lengths (with at least 5 mm. of stem projecting from either end) slight decreases in length of the regenerate were observed with increasing length of the tubes.

4. It was concluded that the powerful effects observed when the distal ends of the stems projected less than 5 mm. from the tubes represent the limitation of diffusion of inhibitors out of as well as of oxygen into the stems. On the other hand, in the light of other experiments, it was suggested that the effects obtained by covering the middle of the

stems represent the extent to which inhibitors of regeneration are eliminated through the unbroken perisarc rather than any induced alteration of the oxygen content of the coelenteron.

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THE NUTRITIVE REQUIREMENTS OF TENEBRIO MOLITOR LARVAE [1]

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A comprehensive review of the literature on invertebrate nutrition was published by Trager (1941). Our interest was stimulated by the possibility that the nutritive response of an insect, caused by a specific biological substance, might be used as a method of assay that could be applied to small quantities of human blood, urine, and necropsy tissue. We have worked intensively upon the problem for several years and our initial studies, started in 1931, were devoted to selecting the insect and the nutritive response best suited for this type of investigation. We chose the growth response of *Tenebrio molitor* larvae for several reasons. First, the larvae attain mature size without access to water which would allow yeast and bacteria to grow in the diet and vitiate results. Second, the larvae are docile, hardy, and large enough to be weighed without escape or injury. Third, the imagos are sufficiently prolific that large numbers of immature larvae of the same age and weight are available for use.

Experimental Methods

A sufficient number of imagos are collected in early summer to insure enough larvae to carry out the planned experiments. We obtained more than 500,000 larvae in 10 weeks from 3,000 beetles, with the following technique: the bottom of a five-gallon can is covered with paper and two pieces of paper folded as fans are placed in the can. Two hundred grams of beetle diet, consisting of white flour and 5 per cent brewer's yeast, is scattered over the fans which are covered with cardboard. A moistened bath sponge, on a glass plate, is placed on the cardboard to supply water for the beetles. Fifteen hundred beetles are placed in each can. The procedure is repeated daily in order to maintain a constant supply of immature larvae of approximately the same age. The growth of stock larvae can be arrested either by feeding them an insufficient diet or by non-hygroscopic refrigeration and such larvae are available throughout the winter months.

[1] This investigation was supported by a Landon Research grant.

The Kerr one-half pint fruit jar is ideal for a feeding cage. The dry dietary ingredients can be sealed in the jar with three or four marbles and numerous jars placed in a rotating device which mixes the diets thoroughly. The diets can be kept sealed from contamination until the experiment is started, when marbles are removed, larvae added, and wire screen and cloth substituted for the self-sealing lid. This makes it possible to study many variables with the same larval colony, groups of which can be fed the different diets at the same time.

The experimental procedure is simple. Immature larvae of the same age and weight are divided into groups of 25. Each group is fed 15 grams of diet [2] and each diet is fed to three groups to obtain a triple check on results. After a sufficient feeding period each group is weighed and the average weight of surviving larvae computed. At ordinary laboratory temperature and humidity alterations in growth are apparent after four to six weeks but maximal alterations are seen after 10 to 12 weeks.

Preliminary Experiments

Early studies, carried out before crystalline nutritive elements were available, demonstrated that larval growth was influenced by many physical and dietary factors and numerous experiments were done to establish a method of procedure that would control the different variables. It was observed that growth was modified by alterations of temperature and humidity; age, refrigeration, and state of nutrition of test larvae; the subjection of the diet to excess heat or chemical manipulation; the growth of yeast or bacteria in the diet; and the relative proportion of fat, carbohydrate, and protein in the diet. Optimum growth occurs when the diet contains not less than 50 per cent carbohydrate and not less than 15 per cent or more than 25 per cent protein. Apparently fats are not required and when present in excess of 3 per cent inhibit growth. These latter observations confirm the findings of Lafon and Teissier (1939) who studied the effect of qualitative and quantitative additions of lipids, carbohydrates, and proteins to the diet of molitor larvae. Tables I and II present some pertinent findings of additional experiments. The results appearing in Table I represent typical observations made at different times when molitor larvae were fed various diets. Animal tissues added to the diets were dried at 70° C. and powdered.

Table I shows that neither white wheat flour nor an orthodox vitamin-free diet allows appreciable growth but optimum growth oc-

[2] Fifteen grams of diet, adequate in nutritional factors, is sufficient to allow 25 larvae to attain adult size, although to carry this number through the life cycle requires 25 to 30 grams.

curs when either of these diets is supplemented with yeast. The results indicate that the vitamin-free diet and yeast causes better growth than white flour and yeast, suggesting that some ingredient of the vitamin-

TABLE I

The effect of different diets upon growth of molitor larvae. Each diet was fed to 75 larvae for 12 weeks. Average initial weight was 2.5 to 3 mg.

	Number of surviving larvae	Average weight (Milligrams)
White wheat flour	42	9
[1] Vitamin free diet	27	6
Whole wheat flour	57	31
White wheat flour and beef blood (5%)	30	4
White wheat flour and beef muscle (10%)	55	20
White wheat flour and beef liver (10%)	73	71
White wheat flour and brewer's yeast (10%)	69	92
Vitamin free diet and brewer's yeast (10%)	60	142

[1] The vitamin free diet contained casein 20 per cent, corn starch 74.5 per cent, Osbourne and Mendel salt mixture 4 per cent, cholesterol 1 per cent and crisco 0.5 per cent.

TABLE II

The influence of different percentages of five separate strains of brewer's yeast on growth of molitor larvae. Each diet was fed to 75 larvae for 83 days. The average initial weight was 2.5 mg.

Per cent of yeast in the diet [1]	Strains of brewer's yeast [2]									
	No. 1		No. 2		No. 3		No. 4		No. 5	
	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)	Surviving larvae	Average weight (mg.)
0.5	47	22	44	23	53	19	38	17	41	14
1.0	52	40	42	40	60	24	43	23	42	19
1.5	62	53	54	48	60	37	52	30	40	23
2.0	56	74	40	68	51	49	48	35	37	27
2.5	54	95	49	80	61	64	50	42	38	37
3.0	58	107	54	92	57	85	53	49	53	43
5.0	47	135	43	126	54	109	54	94	47	78
10.0	58	132	57	129	57	112	52	132	52	105

[1] Control groups fed only white wheat flour averaged 9 mg. in weight.
[2] Supplied by Anheuser-Busch Biological Laboratories, St. Louis, Missouri.

free diet modifies growth. Whole wheat flour allows moderate growth, indicating that the pericarp supplies growth factors. With a basal diet of white flour, skeletal muscle causes a moderate response comparable to larvae fed whole wheat flour. Liver causes better growth

than muscle, which signifies that it contains more growth factors. The addition of blood to the diet retards growth. Yeast causes better growth than liver, indicating that it contains more growth factors or that liver contains substances which inhibit growth. The effect of adding different tissues to a vitamin-free diet has not been studied. A basic diet of white flour was used in preliminary studies because it more nearly imitated the natural food of this insect and survival on inadequate diets was observed to be better.

The observations appearing in Table II were made on a colony of molitor larvae fed white flour supplemented with different percentages of five separate strains of brewer's yeast. The table is presented to show that average growth in a controlled experiment is remarkably constant and the uniformity of results indicate that larvae do not select food particles but eat indiscriminately of the diet.

Table II shows a direct correlation between concentration of yeast and growth response, optimum growth occurring when the diets contained 5 per cent to 10 per cent yeast. The strains are numbered in the order of their ability to stimulate growth and the findings demonstrate the sensitivity of larvae to minor changes in the diet introduced by using different strains of yeast.

The above studies show that molitor larvae will attain mature size on a dry diet incompatible with the growth of yeast and bacteria but, in order to control the different variables, each experimental colony must consist of larvae of the same age, weight, and state of nutrition, started on the various diets at the same time, and kept in the same environment. Moreover, the diets should receive identical chemical and physical manipulation and contain the proper balance of fat, carbohydrate, and protein for optimum growth. Each diet should be fed to 75 or more larvae since there is marked variation in individual growth. When this procedure is followed the only factors which modify growth are the nutritive elements in the diet. However, before larval growth could be used to assay the nutritive elements it was necessary to establish the specific influence of a given factor. This proved to be difficult and was accomplished only after most of the crystalline B components were made available.

Experiments with Synthetic Diets

These studies were undertaken in an attempt to ascertain which of the known purified and crystalline vitamins influenced growth when added singly to a purified diet,[3] which ones stimulated growth by acting

[3] The basal diet contained vitamin free casein 20 per cent, corn starch 74.5 per cent, Osborne and Mendel salt mixture 4 per cent, cholesterol 1 per cent and crisco 0.5 per cent.

in conjunction with other vitamins, and which ones failed to affect growth entirely. We have carried out several experiments on this problem, extending the study to include each new crystalline vitamin when it was available. The source and quantity of vitamins added to the diets used in all experiments appear in Table III.

TABLE III

The quantity and nature of vitamins added to the diets

Nature of the vitamins [1]	Amount per gram of diet [2]
Vitamin A	
Natural purified vitamin from fish liver oil	40 units
Vitamin D_2	
Irradiated ergosterol	7 units
Vitamin E	
Synthetic alfa tocopherol	33 micrograms
Vitamin K	
Crystalline 2-methyl-1, 4-naphthoquinone	20 micrograms
Vitamin C	
Crystalline l-ascorbic acid	100 micrograms
Crystalline choline hydrochloride	100 micrograms
Crystalline thiamine hydrochloride	10 micrograms
Crystalline riboflavin	10 micrograms
Crystalline pyridoxin	10 micrograms
Crystalline calcium pantothenate	10 micrograms
Crystalline nicotinic acid	30 micrograms
Labco flavin free rice polish concentrate Rich in thiamine, pyridoxin, nicotinic acid, and filtrate factors	50 milligrams
Brewer's yeast. The yeast assayed 50 International B_1 units and 50 Bourquin-Sherman G units per gram	50 milligrams

[1] Solutions of crystalline vitamins of known concentration were mixed with the vitamin free diet until a syrup resulted. This was desiccated and powdered in a ball mill.

[2] The quantity of vitamins added to the diets was arbitrary. Five per cent yeast causes optimum growth and the amount of vitamins present in this amount of yeast is much less than the quantity of purified vitamins added to the diets.

Experimental procedure: Various diets, incorporating singly and collectively the purified vitamins, were fed to groups of 75 larvae for 12 weeks. For the sake of brevity only the pertinent results of composite experiments will be presented. These appear in Table IV.

Table IV shows that no vitamin, by itself, promotes growth when added to a vitamin-free diet. Furthermore, vitamins A, D, C, E, K, and choline, failed to influence growth when added to diets containing all the synthetic B vitamins. Moreover, no combination of the B vitamins (thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid) affect growth appreciably unless all five are present.

Finally, growth on diets containing the five B vitamins is only one-third that of larvae fed the vitamin-free diet supplemented with liver or yeast. Table IV also shows that the only vitamin which increased the incidence of survival was riboflavin.

The above findings demonstrate conclusively that molitor larvae require thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic

TABLE IV

The vitamin requirements of molitor larvae. Each diet was fed to 75 larvae for 12 weeks. Average initial weight was 4 to 5 mg.

Diet (Vitamins added to a vitamin free basal diet)	Number of surviving larvae	Average weight (milligrams)
Vitamin A	21	7
Vitamin D	27	6
Vitamin C	19	7
Vitamin E	18	8
Vitamin K	22	7
Choline	23	6
Thiamine (B_1)	27	7
Riboflavin (B_2)	60	8
Pyridoxin (B_6)	21	8
Nicotinic acid (N. A.)	17	8
Calcium pantothenate (C. P.)	25	7
A, D, C, E, K, and choline	22	9
B_1, B_2, B_6, and N. A.	62	13
B_1, B_2, B_6, and C. P.	59	12
B_1, B_2, N. A., and C. P.	65	14
B_1, B_6, N. A., and C. P.	24	11
B_2, B_6, N. A., and C. P.	69	14
B_1, B_2, B_6, N. A., and C. P.	73	36
A, D, C, E, K, choline, B_1, B_2, B_6, N. A., and C. P.	68	36
Brewer's yeast	73	114
Rat liver	67	102
Vitamin free basal diet	25	7

acid, however, these vitamins could only stimulate growth by acting in conjunction with each other. It was thought that superior growth on yeast or liver might be caused by phosphorylated flavin and the influence of lactoflavin phosphate [4] was investigated to test this concept. Two experiments were carried out with the same controlled colony of larvae; in the first, graded amounts of lactoflavin phosphate and riboflavin were each added to an unsupplemented vitamin-free diet, and in the second, the two flavins were added to the basal diet further supplemented with vitamins A, D, C, E, thiamine, pyridoxin, nico-

[4] The lactoflavin phosphate was obtained through the courtesy of Dr. R. Kuhn of Heidelberg. The preparation was made according to the method published by Kuhn, Rudy, and Weygand (1936).

tinic acid, and flavin-free rice polish concentrate as a source of pantothenic acid. The various diets were each fed to 75 larvae for 81 days and the results appear in Figure 1.

Figure 1 shows that results with the unsupplemented vitamin-free diet were entirely negative and confirm previous observations that riboflavin can only stimulate growth by acting in conjunction with

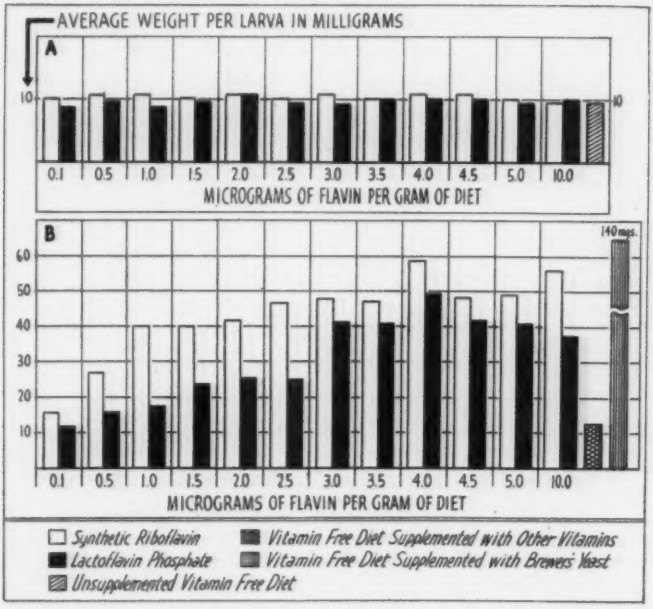

FIGURE 1. The comparative effect of synthetic riboflavin and lactoflavin phosphate upon growth of molitor larvae. Each diet was fed to 75 larvae for 81 days. The average initial weight was 4.9 mg.

A. An unsupplemented vitamin free diet was used.

B. The basal diet was supplemented with vitamins A, D, C, E, thiamine, pyridoxin, nicotinic acid and flavin free rice polish concentrate.

other components of the vitamin B complex. The findings demonstrate that lactoflavin phosphate possesses no merit over synthetic riboflavin in this respect.

In the second experiment results were different. When the basal diet was supplemented with the other essential B vitamins the influence of riboflavin is apparent. The figure shows that maximal growth was caused by approximately one microgram of riboflavin and 2.5 to 3 micrograms of lactoflavin phosphate, per gram of diet, demonstrating

that flavin phosphate, together with the other essential vitamins, causes no better growth than riboflavin. Indeed, the results suggest that excessive amounts of lactoflavin phosphate retard growth. Additional observations, not included in this report, show that diets containing as much as 75 micrograms of riboflavin per gram cause no inhibition, therefore the retarding effect of lactoflavin phosphate appears to be due to the phosphoric acid radical. The lesser potency of lactoflavin phosphate is logically explained on the basis of inert phosphoric acid content. The study was not designed to measure precisely the amount of flavin required for maximal growth but Figure 1 shows that larvae are sensitive to minute amounts and it appears that maximal growth results when the diet contains one to 3 micrograms per gram.

The experiments show that superior growth on diets containing yeast (See Table IV) is not due to the fact that zymoflavin occurs as the phosphoric acid ester and the finding indicates that yeast and liver contain larval growth factors additional to the vitamins investigated.

Comment

The vitamin requirements of several orders of insects have appeared in the literature but reports are so variable that no positive conclusions can be drawn as to which factors are essential. Apparently the only crystalline vitamins required are included in the vitamin B complex but there is no agreement as to specificity. Some of the confusion may be due to the different orders investigated. Subbarow and Trager (1940) reported studies with mosquito larvae that are sufficiently related to our findings to warrant comment. They state that these larvae require thiamine, riboflavin, pyridoxin, pantothenic acid probably glutathione and nicotinic amide, and certainly other unknown substances present in yeast and liver extract. Although we used a different experimental approach on an unrelated order of insect, we obtained identical results. It appears, therefore, that all insects probably have similar basic nutritive requirements and conflicting results of others are possibly due to the common practice of using highly purified diets in nutritional experiments. The failure of a given dietary factor to influence the nutritive response, when added to such diets, has frequently been interpreted as evidence that the test animal either does not require the vitamin or can synthesize it. We believe our studies with molitor larvae demonstrate the fallacy of this concept since no vitamin, by itself, had any effect and growth was not appreciably stimulated until thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid were all present. Even then

growth was less than one-third optimum and it is possible that with more complete diets the specific effect of additional crystalline vitamins could be demonstrated. Moreover, our studies were limited to larval growth and additional vitamins are probably necessary for other nutritive responses such as survival, metamorphosis, pupation, and fertility.

It is apparent that the nutrition of insects is extremely complex and that numerous factors, known and unknown, are required for optimum effects. However, with the rapid advances being made in the chemistry of nutrition and biology, it appears entirely possible that they may ultimately be reared on completely synthetic diets. Our studies still leave much to be learned but we believe they are sufficiently complete to demonstrate the cardinal advantages of molitor larvae as test material for this major problem. In some of our experiments more than 100 variables were investigated with a single colony of thousands of larvae, of the same age and weight, which were started on the various diets at the same time. Furthermore, it is possible to carry out such experiments with maximal economy of time, space, and expense.

It is interesting to speculate on the reason why so many of the B vitamins had to be in the diet before growth occurred and why other purified vitamins caused no effect. It is perhaps significant that most of the B vitamins are now known to function by entering into cellular enzyme systems, and it may be that insects require only the "enzymatic" vitamins. If this is true, thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid, as well as additional factors required for optimum growth, function in this manner. It is possible, therefore, that insects may have unique importance as test material in the elucidation of this problem.

Although larval growth is dependent upon the interrelationship of many factors, the effect of any one factor is concise and specific provided the complementing factors are added to the basal diet. Molitor growth, therefore, can be used as a biological assay for any isolated factor whose specific influence can be demonstrated. At present these factors include thiamine, riboflavin, pyridoxin, nicotinic acid, and pantothenic acid. These assays can be both qualitative and quantitative on any material that does not contain the unknown factors required for optimum growth. Such assays may be of practical interest to the synthetic chemist who is interested in studying the biological activity of compounds which are related to the above vitamins. Furthermore, they may be important to the pharmacologist who is interested in the control assay of pharmaceuticals consisting of the above nutritive factors.

Summary

1. A standard experimental method is presented which can be used to investigate factors that modify the nutritive responses of molitor larvae.

2. Larvae failed to grow when fed a vitamin-free diet of casein, fat, carbohydrate, salt mixture and cholesterol but, when this diet was supplemented with yeast or liver, optimum growth occurred.

3. When the above diet was supplemented, individually, with vitamins A, D, C, E, K, choline, thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid, negative results were obtained. However, when added collectively to the diet, approximately one-third optimum growth resulted.

4. When different combinations of the above vitamins were added to the diet, no appreciable growth occurred unless the diet contained thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid. Conversely, if any of these five factors was omitted, results were essentially negative. Vitamins A, D, C, E, K and choline had no effect.

5. On the assumption that optimum growth on yeast might be caused by phosphorylated flavin, lactoflavin phosphate was investigated. The phosphorylated flavin caused no better growth than synthetic riboflavin, indicating that yeast contains larval growth factors additional to the above vitamins. Biotin, paraminobenzoic acid, folic acid and inositol were not studied.

6. Molitor growth is suggested as a method of biological assay for thiamine, riboflavin, pyridoxin, nicotinic acid and pantothenic acid.

Acknowledgment

We are deeply grateful to Merck and Company, and Eli Lilly and Company, who generously supplied us with purified nutritive factors when only a small amount of such material was available.

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THE CILIARY TRANSPORT-SYSTEM OF ASTERIAS FORBESI

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Certain persistent tubular structures derived from the anterior and right-posterior enterocoels of the larvae of echinoderms, and traditionally termed 'perihaemal systems,' seem questionably so-named; for they assuredly do not in major manner function as haemal, or blood, systems do in other phyla. If they play any part as a transport system serving nutritional, respiratory, and excretory ends, it must be a minor contribution indeed; but exclusive of the echinoderms, even animals of relatively minute size, e.g., tiny annelids, or semi-visible arthropods of different sorts, are generally equipped with pulsatile or tubular organs, or both, of constant anatomical make-up and relations which serve the function of transport. In short, it is probably true that echinoderms are the largest animal organisms lacking the homologue of a vascular system as that designation is customarily used.

In most if not all echinoderms, media serving respiratory, excretory, and nutritional ends are propelled by cilia on external, in coelomic, and in other, e.g., endodermally lined, spaces. In as much as the circulation paths thus established by ciliated tracts are for the most part very definite and constant as between individuals of the same species, the mapping of these paths is a procedure in concrete anatomy.

It should be mentioned at once that Gemmill (1915) published the results of his study of ciliary tracts in several asterids common in English waters, but apparently did not use *Asterias forbesi*. Furthermore, Gilsen, who published his extensive paper on echinoderms in 1924, covering all classes of that phylum, did not include this species; also, so far as ciliation is concerned, his account is limited to ciliation of external epithelia. Other less extensive studies are cited later in this narrative.

The purpose of the present paper, therefore, is to record observations on the cilia-produced flow on external and internal epithelia of the starfish most frequently employed as a study-type in all grades of educational institutions east of the Rocky Mountains in the United States. A detail of the mechanism serving certain physiological processes in this asterid may thus be more available.

METHODS

All conclusions as to flow-direction were made by use of carmine suspension in sea-water, introduced to the area under observation from a small-bore pipette, and following its movement with a binocular dis-

secting microscope. It was assumed that the action of cilia was not modified by extirpation of the area on which they were located.

Ciliary tracts on external surfaces [1]

1. General aboral areas:

Echinoderm skeletons are mesodermal, and ectodermal epithelium completely covers the body exterior, though often reduced on tips of spines. While the epithelia of spines show their more-or-less individual ciliary equipment (described later), the composite directional flow on the body surface as a whole is shown in Plate I, Figure 1; it is centrifugal from the central disc (anal area) to the distal ends of the rays, with currents running laterally (inter-radially) from the axial mid-lines. The only areas not conforming to this rule are those in the angles of the inter-radial surfaces; here the flow is centripetal, with the obvious result that gonadal products emerging in those angles are thrown into the open, not into the substrate.

2. Spines and branchiae:

Cilia on aboral spines and branchiae uniformly beat from the bases to the distal ends on all sides, save that on all there is deviation enough (rarely with spiral tendency) so that there is a resultant mass-effect on the ray-surface-as-a-whole of a sort already mentioned.

3. Pedicellariae:

Figure 1 indicates the ciliary direction-map for a pedicellaria, the forceps and scissors types being similar. A specially adapted area is

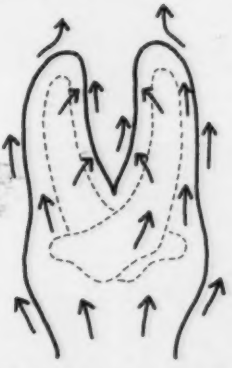

FIGURE 1. Ciliary flow on surface of a pedicellaria.

[1] Photographs reproduced in this paper were made by Arthur L. Princehorn, head of Photographic Service in Oberlin College.

that on the apposed sides of the jaws; cilia sweep suspended particles onto the inter-jaw ridges, i.e. onto the biting area, on which in turn the flow is distad.

4. The madreporite:

Madreporitic surfaces show currents which, in the main, flow toward the aboral disc-center, with minor flows toward adjacent rays. About 50 per cent of specimens show considerable streaming in all

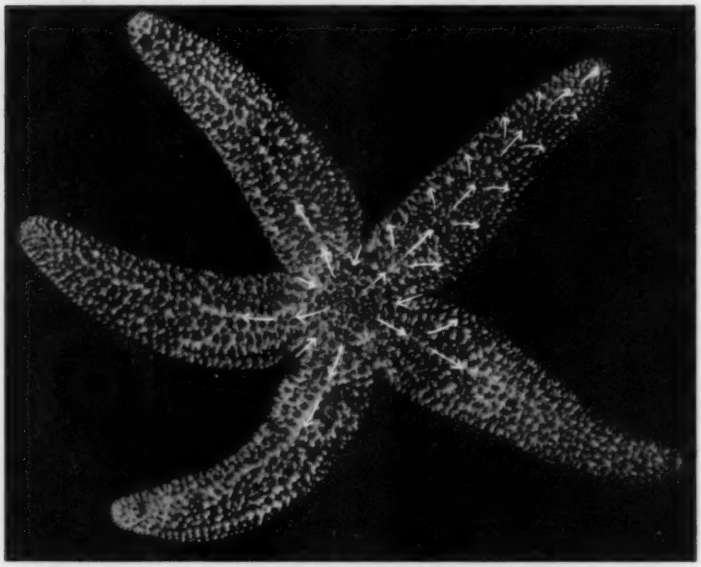

PLATE I, FIGURE 1: Direction of ciliary currents on external aboral surface.

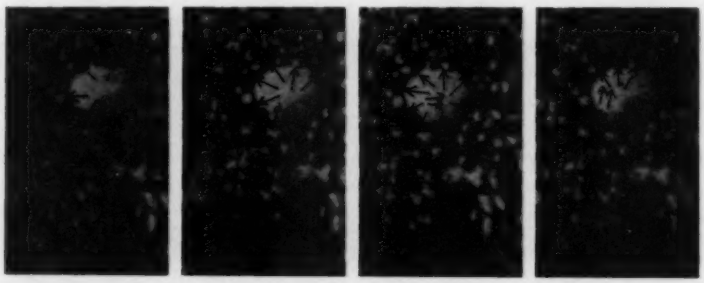

PLATE I, FIGURE 2: Various flow-patterns on madreporic plates.

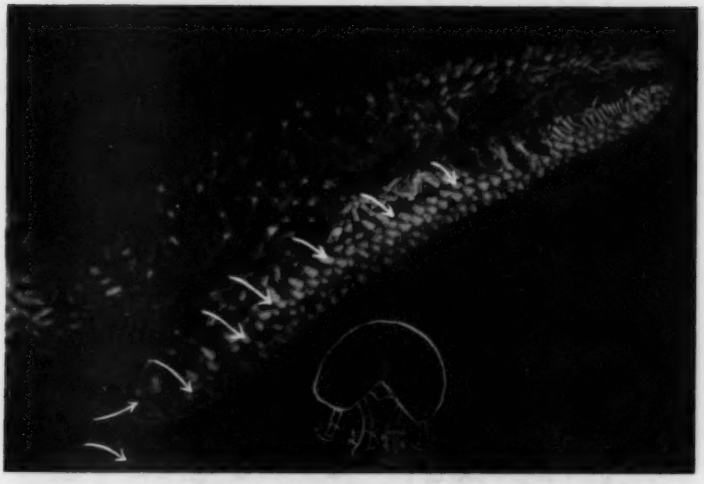

PLATE I, FIGURE 3: Ciliary currents on oral surface of one ray. Insert is diagram of cross-section of ray to show flow-direction on tube-feet and marginal spines.

directions from the porite center; as madreporitic plates themselves have a varied structural pattern, this ununiformity is not surprising. Plate I, Figure 2 presents instances of observed variations. Cilia in the pore-canals of a plate have a positive outward beat; it is this fact, presumably, which seems to have influenced Hartog (1887) to believe erroneously that a considerable portion of cilia in the stone-canal itself beat similarly.

5. General oral surface:

The ciliary-flow picture of a sample ray of this area is shown in Plate I, Figure 3; currents run centrifugally from the median oral region, but take an inter-radial direction from the ambulacral grooves, tube-feet, and adjacent ray surfaces.

6. Spines and branchiae (oral aspect):

On the lateral ray areas these structures are equipped like those on the aboral surface, viz. currents run distally. Those on the margins of the ambulacral grooves, however, are clothed with cilia which sweep laterally, i.e. at right angles to the long axis of spine or branchia, transversely around each side toward the inter-radial space. This description holds true for ciliation on either side of the groove (Plate I, Figure 3).

7. Tube-feet:

On these the set-up is the same as just mentioned for spines and branchiae on ambulacral margins, i.e. at right angles to long axis around toward the inter-radial space. Either side of the groove has a flow pattern which is the mirror image of that on the other side of the axial midline (Plate I, Figure 3).

8. Ambulacral groove:

Ciliation in the bottom of the groove is very weak indeed, with uncertain tendency to carry particles centripetally. The flow on either side of the midline is strongly toward the inter-radial space of its side. Neighboring cilia on tube-feet, unless these are completely eliminated, confuse observations.

9. Perioral membrane:

Cleared of near-by structures, all currents here flow toward the mouth and thus contribute, though in immeasurable degree, to nutritional intake.

Physiological Adaptations of External Ciliation

1. Material in suspension tending to settle on the surface and interfere with respiration or excretion is swept away by the shortest path; anal expulsions are similarly dispersed.

2. Cilia on the madreporite beat in such manner as to prevent debris from clogging the entrance to the water-vascular system.

3. Materials handled by pedicellaria cilia are brought from lateral contacts into their 'bite' area.

4. Cilia in ambulacral grooves, on tube-feet, and on groove margins propel the flow inter-radially, save in the midline of the groove where movement is uncertainly oralward.

5. Centrifugal flow, plus that in aboral direction in inter-radial angles, prevents gonadal products from settling on the relatively congested oral surface, where they might be engulfed in the food-flow, find poor oxygen supply, or be buried in the substrate.

Ciliary Tracts on Internal Surfaces

A. The major perivisceral coelom and organs covered by its peritoneum.

Food absorbed through gastric walls (if this occurs) or their diverticula, as well as oxygen from branchial structures, depends entirely, or very nearly so, on ciliary propulsion for distribution to tissues not parts of those systems. Every coelomic surface is ciliated. For the rays, in general, all flow is centrifugal (distad) except that on the

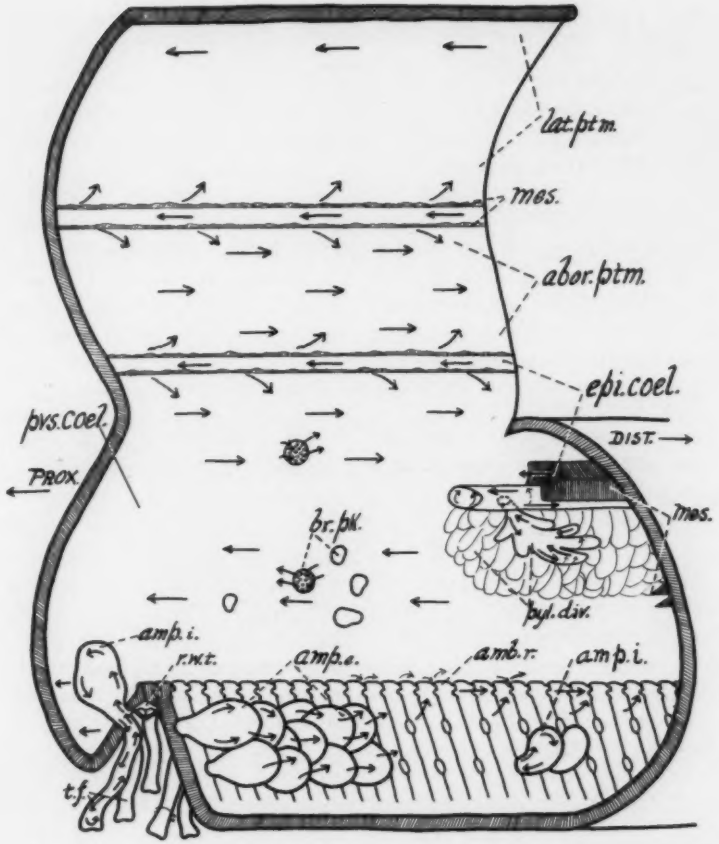

FIGURE 2: Stereogram of structures in opened ray of starfish, with direction of ciliary flows on same:

abor. ptm.	aboral peritoneum
amb. r.	ambulacral ridge
amp. e.	ampulla (exterior)
amp. i.	ampulla (interior)
br. pk.	branchial pocket
DIST.	distal direction
epi. coel.	epigastric coelom
lat. ptm.	lateral peritoneum
mes.	mesentery
PROX.	proximal direction
pvs. coel.	perivisceral coelom
pyl. div.	pyloric diverticulum
r.w.t.	radial water-tube
t.f.	tube-foot.

lateral (inter-radial) peritoneum. A certain degree of movement of coelomic contents is effected by the slow bending of rays but such would seem to be quite incidental. Certain details as to perivisceral coelomic ciliation are:

1. On the aboral body-wall the direction is distad, save in the narrow epigastric spaces between the mesenteries supporting the pyloric diverticula, where it is centripetal; there is some laterad flow from the inter-radial member, and mediad flow from medial member of these mesenterial pairs (Figure 2, and Plate II, Figure 1).

2. The cilia-propelled flow on the pyloric diverticula ('hepatic lobes') is the same on both oral and aboral surfaces, viz. distally along the median axial line of each of the two lobes; also distally along axes of the lateral lobular components and on each of their out-pocketings (Plate II, Figure 2).

3. Cilia on the cardiac stomach retractor muscles beat centrifugally (Plate II, Figure 3).

4. A strong centripetal flow occurs on the epithelial lining of the inter-radial body wall (Plate II, Figure 3; Figure 2).

5. On the ambulacral ridges the flow runs distally, the stream dipping into and out of the inter-ossicular depressions (Figure 2).

6. On the lateral walls of ambulacral ridges the flow is from the ampullary area toward the crest of the ridge (Figure 2; Plate II, Figure 3).

7. The currents on the peritoneal covering of the gonad on the aboral aspect are distad on the main axis and on the subdivisions of the organ as they extend toward the ambulacral ridge (Plate II, Figure 3). On the oral surfaces ciliary propulsion is centripetal (proximad), the reverse of that on the aboral area, thus supplementing that on the inter-radial lining of the ray coelom.

8. The flow on all coelomic surfaces of the stomach (cardiac and pyloric) is aboralward from the perioral region; that on the perioral membrane is weak and irregular.

9. Several branchiae arise externally from each internal branchial pocket; the flow into and out of these pockets conforms, as to direction, with that on the area where each is located (Figure 2).

10. Each branchia has a densely ciliated lining, the flow on all sides being toward the distal end; the stream out of a branchia is down its axial center, forced thus by inflow on all sides.

11. The junction between the rays and central disc, with the inter-radial pillars, ambulacral ridge origins, and Tiedemann's bodies, presents a somewhat complex current pattern, easiest appreciated from a

PLATE II, FIGURE 1: Currents on peritoneal lining of aboral body-wall of one ray. Dotted lines indicate attachments of mesenteries supporting pyloric diverticula. Currents dip into and out of branchial pockets.

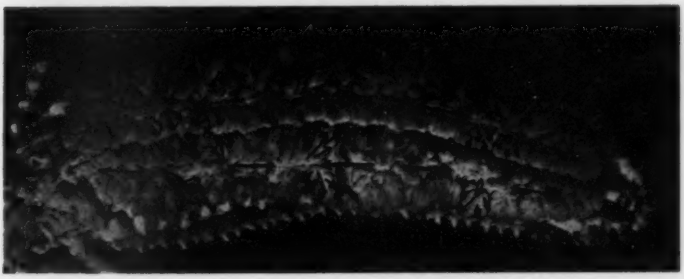

PLATE II, FIGURE 2: Aboral surface of pyloric diverticula in one ray. Currents are distad on all diverticular components.

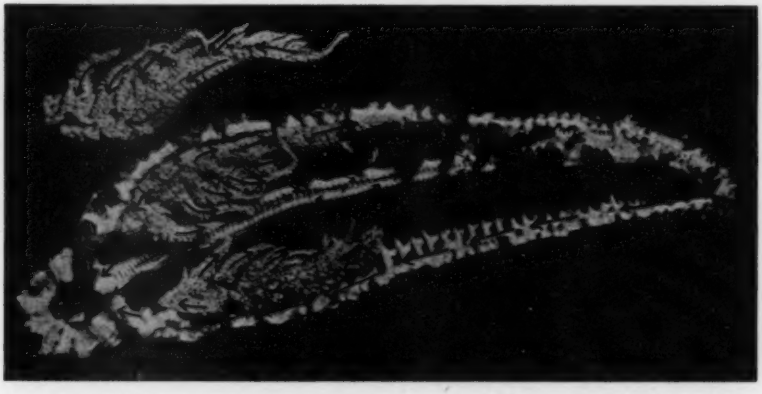

PLATE II, FIGURE 3: Ciliary currents on surface of structures in single ray with diverticula removed. The smaller figure shows the oral aspect of a gonad.

diagram (Figure 3). Direction flow on each side of an ambulacral ridge, i.e. between it and the ambulacral (inter-radial) pillars, is centripetal. On each Tiedemann's body the aboral ciliation beats toward the inter-radius to which it is adjacent. As this flow passes

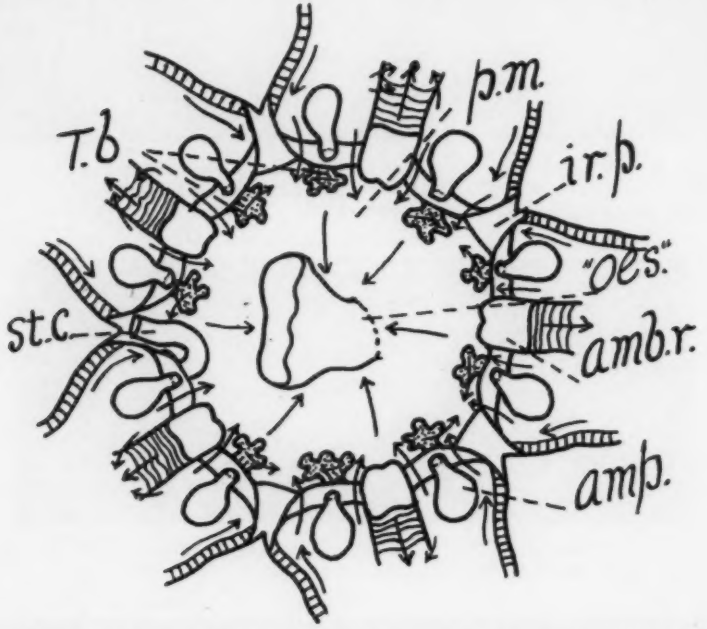

FIGURE 3: Diagram of structures located at margin of perioral membrane, and bases of rays, internally; direction of ciliary currents on same indicated.

amb. r.	ambulacral ridge
amp.	ampulla
ir. p.	inter-radial pillar
"oes."	oesophagus
p. m.	perioral membrane
st. c.	stone canal
T. b.	Tiedemann's bodies.

equatorially around the body, its direction is reversed on the oral aspect.

12. Currents on the peritoneal fold surrounding the stone canal and axial organ run across the long axis of these organs, in counter-clockwise direction when viewed from what might be termed the gastric side of these structures; however, at the junction of this fold with the body wall there may be currents running oralwards.

B. The peritoneum of the epigastric coelom.

1. The central area of this space, as exposed by removal of the pyloric stomach, shows a rather unexpected ciliary behavior. Viewed from its interior and looking aboralward, currents run in counter-clockwise direction, the flow on the rectal caecum supplementing the rest, i.e. going in the same direction. The flow into and out of branchial pockets conforms to this directional pattern also. There are minor deviations on small areas but the composite effect is as mentioned.

The writer is at a loss to account for this exception to what is otherwise, for the most part, quite a symmetrically ordered anatomy. However, one may appeal to the fact that the epigastric coelom, though centrally located in adult starfish, arises embryologically from the right posterior enterocoel, and explain this circular movement as a one-way ciliary beat modified when incorporated into a middle, polar position. No such one-way retention of current direction is found in the adult disposition of tracts on the left posterior enterocoelar (major perivisceral) peritoneum, however.

2. Ciliary movements on the oral face of the rectal caecum are noted above; those on the aboral surface are mixed, but with a tendency to flow distally on each lobe.

3. In the extensions of the epigastric coelom between the two mesenteries suspending each main lobe of the pyloric diverticula the contained fluid is swept strongly centripetally (Figure 2; Plate II, Figure 1).

C. Lining epithelium of the water-vascular system.

1. Stone canal: All epithelial surfaces here show a strong inflow current, if any. As is well known, a prominent infold extends most of the length of the canal along one side, its free edge giving rise to symmetrically arranged, coiled extensions which in cross-section present a double volute pattern. No positive movement of suspended particles in contact with the convex faces of this bifid structure appears, but their concave surfaces (which are continuous with the rest of the canal peritoneum) present a strong inwardly-going (oralward) current.

The fact that the stone-canal and sieve-plate are the main structural features between the ambulacral system and the exterior has always presented a temptation to assume that both inflow and outflow of water is effected by a mechanism in the stone-canal and its associated structures. However, it has long and repeatedly been recognized (Ludwig, 1890; Cuenot, 1891; McBride, 1896; Gemmill 1914) that the ciliated surfaces present only inward (oralward) currents. It seems highly probable, therefore, that outflow from the water-vascular spaces is

brought about by spasmodic contractions of the muscular walls of the tube-feet and associated ampullae.

2. The ampullae and tube-feet: All epithelia lining these structures are strongly ciliated for carrying water into and out of their cavities. By reference to Figure 2, the facts are at once appreciated: from the radial canal the current runs *into* the ampullae on the aboral side; *outward* on the oral area; *into* the tube-feet on the side toward the ambulacral groove margins; *out of* the tube-feet on the side toward the ambulacral midline.

D. Endodermally lined spaces.

1. From oral to anal ends of the alimentary system the epithelial cilia beat aboralward, as would be expected.

2. The ducts from the pyloric diverticula show a two-way flow, i.e. centripetal on the aboral area, centrifugal on oral surfaces (Figure 2). This is further true of ducts in the subordinate spaces of the organ. This parallels Gemmill's (1915) observations on several asterid forms and what Irving (1924) noted in our west coast Patiria. The diverticula are the main places of food absorption as well as of enzyme secretion, as long ago noted by Cuenot (1901) and Cohnheim (1901), and more recently proved by Jordan (1913), van der Heyde (1923) and Irving (1924); and this equipment of cilia seems perfectly adapted for bringing food from the stomach to the place of its subsequent digestion and utilization. Minor hoop-like tracts connect the in-coming (oral) currents with the out-going (aboral) in every diverticular lumen (Figure 2).

3. The endothelium of other alimentary diverticula, in particular the rectal caecum, is so given to mucus secretion that no distinct ciliary movement is detectable in it. Filling and emptying is rhythmical (Budington, 1936), the former accomplished by (a) relaxation of its own walls, and (b) the pressure of fluids in the pyloric stomach, thence through the intestine and into the caecum. Emptying of the rectal caecum is due to muscular contraction of its own walls.

Discussion

As already mentioned, thorough observation of asterid ciliation on several types was made by Gemmill (1915). He worked on ***Asterias rubens***, ***Astropecten irregularis***, ***Porania pulvillus*** and ***Solaster papposus***. It has been my privilege to examine these same forms, also ***Luidia sarsi***, ***Asterina gibbosa***, and ***Henricia sanguinolenta***. As would be anticipated, there is extensive agreement as to fundamental ciliary flow pattern in particular species belonging to the same genus; but to illustrate what seem species differences in the genus Asterias, the following table of

contrasts between *A. rubens* (following Gemmill's account) and *A. forbesi* is offered. Except for the items mentioned, they agree closely though not absolutely.

Area (external)	*A. rubens*	*A. forbesi*
Inter-radial aboral surfaces of rays	Confused	Strongly inter-radial
Aboral aspect of disc	Confused	Strongly centrifugal
Perioral membrane	Centrifugal	Centripetal
Madreporite	Toward center	Generally away from center, with tendency toward aboral flow all over; much variation
Gills and spines	Spirally to free ends	Same result but lack spiral feature
Tube-feet	Toward free end spirally	Same result but lack spiral feature
Pedicellaria	To free ends irregularly	Same but with bilateral flow into bite area
Area (internal)		
Lateral ambulacral ridge	Inward or outward, to or from midline	Toward midline only
Gonads	From attached to free extremities	Same on aboral surfaces; reverse on oral surfaces

In the writer's opinion, however, to use ciliary tracts as serious consideration in species identification would be an unwarranted procedure, for, as Kellogg (1915) pointed out in extensive studies of ciliation in lamellibranchs, variations of considerable degree occur commonly within a given species. As a criterion in generic determination, nevertheless, the major ciliary tracts would be as significant for comparison as would any other structural feature.

In its major aspects, then, the transport-system in asterids, while unlike that of metazoa at its own or higher levels, is definitely organized in an essentially constant pattern. No single central propelling organ is present. Instead, a myriad of cilia move environing fluids over ecto-, meso-, and endodermic epithelia in a manner which is comparable, physiologically, to that accomplished by arteries, capillaries, and veins in a majority of higher metazoa. The tracts herewith described as loci of centrifugal currents may easily be compared to arteries: likewise, centripetal flow-tracts may be thought of as analagous to veins.

Functionally the metabolic story in higher animals involves anatomical centralization of external respiration in gills or lungs, of excretion in tubular organs occurring singly or in masses (kidneys), of nutritive absorption in a centrally located food tube. In echinoderms the tissues accomplishing respiration and excretion are markedly non-

centralized; in large measure the same is true of the tissues serving food absorption, for the pyloric caeca, where food dialyzes into the coelom, may well be rated as diffuse also. In consequence, the transfer of anabolic and katabolic substances in asterids is not so much more complicated than in coelenterates and platyhelminths as one might assume. Diffusion through intercellular spaces, abundant in parenchymatous tissues, is adequate for the lower phyla mentioned; with greater specialization of anatomical features and the development of coelomic cavities, heavy ciliation externally and internally in asterid echinoderms is an efficient substitute for any more elaborate system for transport. Its fixed organization into a genetically repeated pattern of tracts makes it as definite as is the tubular vascular system, and other structural constants, in many other invertebrate and all chordate animals.

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